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Bioavailability and chronic toxicity of metal mixtures in freshwater: modelling and implementation in risk assessment

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences

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List of abbreviations

%AFA	Fraction of active fulvic acid
AHA	Aldrich humic acid
AIC	Aikake information criterion
B	Belgium
BC	Before christ
BL	Biotic ligand
BLM	Biotic ligand model
CA	Concentration addition
CA-DRC	Concentration addition-dose response curve
CA-SSD	Concentration addition-species sensitivity distribution
CCME	Canadian council of ministers of the environment
CI	Confidence interval
DIC	Dissolved inorganic carbon
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
DRC	Dose response curve
EC	European commission
ECx	x% effective concentration
EDTA	Ethylene diamine tetraacetic acid
EQS	Environmental quality standard
EU	European Union
F	France
FA	Fulvic acid
FIAM	Free ion activity model
FOREGS	Forum of the European geological surveys directors
GFAAS	Graphite furnace atomic absorption spectrophotometry
GSIM	Gill surface interaction model
HA	Humic acid
HC5	5% Hazardous concentration
IA	Independent action

IA-DRC	Independent action-dose response curve
IA-SSD	Concentration addition-species sensitivity distribution
IC	Inorganic carbon
ICP-OES	Inductive coupled plasma-optical emission spectroscopy
LA50	lethal accumulation at 50% mortality
LOEC	Lowest observed effect concentration
MMBM	Metal mixture bioavailability model
MOPS	3-N-morpholinopropanesulfonic acid
NICA	Non-ideal competitive adsorption
NIST	National Institute of Standards and Technology
NL	The Netherlands
NOEC	No observed effect concentration
NOM	Natural organic matter
OC	Organic carbon
OECD	Organisation for Economic Co-operation and Development
PAF_{Mix}	Predicted affected fraction by the mixture
PEC	Predicted environmental concentration
PNEC	Predicted no-effect concentration
RCR	Risk characterization ratio
REACH	Registration Evaluation and Authorisation of Chemicals
RE	Relative effect
RR	Relative reproduction
SCHER	Scientific Committee on Health and Environmental risks
SSD	Species sensitivity distrubution
TU	Toxic unit
USA	United States of America
USEPA	United States environmental protection agency
WFD	Water framework directive
WHAM	Windermere humic aqueous model
YUT	Yeast-urtica-trout chow

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General introduction and conceptual framework of the study

1. General introduction and conceptual framework of the study

1.1 General properties and classification of Ni, Zn, and Pb

Metals are usually characterised by good electrical and thermal conductive properties, are mostly occurring as cations (positive ions), and have a lustrous appearance (Luoma & Rainbow, 2008). The classification of metals has been intensively debated. Nickel (Ni), zinc (Zn), and lead (Pb) have been often classified as 'heavy metals' and/or 'trace metals'. However, these definitions are not unambiguous and are nowadays considered to be outdated (Hodson 2004). The chemically most correct classification system for metals was proposed by Nieboer and Richardson (1980). These authors classified metals according to their Lewis acid properties. Class A metal ions (Lewis hard acids) tend to bind ionically with ligands. The major cations, such as Na, Mg, Ca, and K, are considered to be Class A metals. Class B metal ions (Lewis soft acids), such as, Ag, Cu(I), Hg, and Au, on the other hand tend to bond covalently with ligands. Ni, Zn, and Pb(II), the metals considered in the present doctoral thesis, have properties that fall in between these two classes and are, therefore, called borderline metals.

The atomic number, atomic weight, density and electron configuration of Zn, Ni and Pb are summarized in Table 1.1. Nickel belongs to the first row of transition metals, while Pb and Zn are considered to be post-transitional metals. Zinc is physically characterised by a good electrical conductance, a relatively low melting and boiling point, and malleability between 100-150°C. Nickel is physically characterized by its magnetic properties at temperatures between -20°C and 30°C, a high ductility, a high resistance against corrosion and a high electrical and thermal conductivity. Lead is characterised by a poor electrical conductivity compared to other metals, a high density, and its ductility and malleability. Ni, Zn, and Pb occur primarily in their +2 oxidation state (Zumdahl 2005).

Table 1.1. Overview of atomic number, atomic weight, density and electron configuration of Zn, Ni and Pb (Zumdahl 2005)

Metal	Atomic number	Atomic weight (g/mol)	Density (g/cm^3)[a]	Electron configuration
Ni	28	58.96	8.90	[Ar] $4s^2$ $3d^8$
Zn	30	65.38	7.14	[Ar] $3d^{10}4s^2$
Pb	82	207.2	11.34	[Xe] $4f^{14}5d^{10}6s^26p^2$

[a] At a temperature of 20°C.

1.2 Nickel, zinc and lead in society and the environment

1.2.1 Historical and current use of Ni

Nickel as an element has only fairly recently been discovered. In 1751 the Swedish chemist Axel Frederik Cronstedt isolated Ni from a niccolite ore which he had mistakenly considered to be a copper ore (Weeks 1932). However, Ni has been used long before due to the resemblance of its ores with silver or copper ores. The first unintentional use of Ni has been dated as far back as 3500 BC. Traces of Ni (up to 2%) have been found in bronzes from Syria. Between 1700 and 1400 BC, copper-nickel and copper-nickel-zinc alloys, in that time described as 'white copper', were used in the manufacturing of weapons, utensils, and other metal ware in ancient China (Nriagu 1980). The actual exploitation of Ni started only in the 19th century, when a technology was developed to separate Ni from impurities in the ores (Sevin 1980). Since then several applications of Ni have been discovered and the world Ni production has increased ever since. The world Ni mine production in 2012 was estimated at 2.18 million tonnes. Today, the most important Ni mining countries are the Philippines, Indonesia, Russia, the USA and Canada. The global demand of primary Ni was estimated at 1.66 million tonnes in 2012 (USGS 2012). Currently, the biggest consumer of primary Ni is China which uses almost half of the global demand (770 000 tonnes in 2012) mainly for stainless steel applications. The total Ni consumption in the countries of the European Union in 2012 was 211 000 tonnes (USGS 2012).

The characteristics of toughness, ductility, high-temperature stability, and elevated corrosion resistance make Ni a valuable metal in today's metal industry (Reck et al. 2008). About 2/3 of worldwide Ni consumption is used in stainless steel, which usually contain 8-12% Ni (

1.1). 20% of Ni consumption is used in other steel and non-ferrous alloys and 9% in plating applications. The remaining 6% of Ni consumption is used for other applications among which coins, electronics, and batteries for portable equipment and hybrid cars (Nickel Institute 2015).

1.2.2 Ni in the aquatic environment

Ni is the 22nd most abundant element in the earth crust. The earth crust contains 0.0075% Ni (Zumdahl, 1995). Nickel occurs naturally in the aquatic environment due to weathering of Ni containing bedrocks, geochemical processes and atmospheric deposition originating from

amongst others soil dust, volcano eruptions, sea sprays and forest fires (Eisler 1998). Anthropogenic sources of Ni in aquatic systems are atmospheric depositions, surface runoff, industrial effluents and waste water treatment facilities (Pyle & Couture 2012). Aquatic dissolved Ni concentrations in pristine regions are usually lower than 10 µg/L (Chau & Kulikovsky-Cordeiro 1995; Luoma & Rainbow 2008), but can be locally higher in regions naturally high in Ni (Chau & Kulikovsky-Cordeiro 1995). For surface waters near industrial sites, dissolved Ni concentrations between 50 and 2000 µg Ni/L have been reported (Chau & Kulikovsky-Cordeiro 1995).

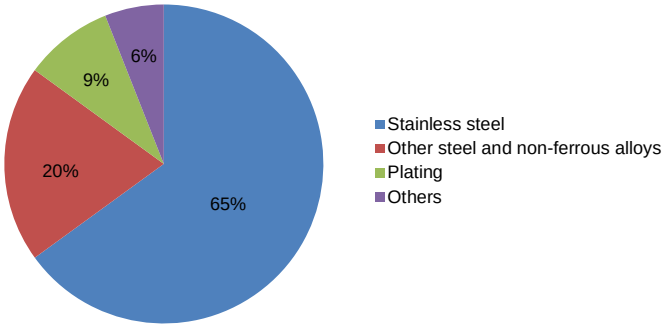

Figure 1.1. Primary uses of Ni (Nickel Institute 2015).

1.2.3 Historical and current use of Zn

Zinc ores have been used for Zn-Cu alloy brass ornaments since 1000 BC in ancient Greece. It was considered in that time as an exotic, expensive metal. The demand in Zn increased in the Roman period, during which Cu-Zn alloys were first used for coin making, but rather rapidly displaced bronze in decorative metalwork (Paddock 1978). However, it was only in the 14th century in India that Zn was really recognized as a separate metal. The knowledge of the Indians on how to smelt zinc ores was carried from there to China. Portuguese traders brought Zn from China to Europe in the 17th century (Weeks 1932). Global demands of Zn increased during the industrial revolution (Nriagu 1996). Today, Zn is the third most used non-ferrous metal worldwide, after aluminium and copper. The global mine production of Zn was estimated in 2014 at 13.5 million tonnes/year. The overall Zn consumption was around 13.7 million tonnes in 2014 (ILZSG 2015). The most important Zn mining countries are China, Australia, Peru and the USA. The most important Zn manufacturing countries are China, the USA, India and the Republic of Korea (USGS 2013a).

The most important application of Zn nowadays is in protecting steel against corrosion by galvanisation (50% of the Zn consumption). Other important applications are the use in the construction industry, in brass and bronze, and in various chemicals (ILZSG 2015). An overview of the today's primary use of Zn is given in Figure 1.2.

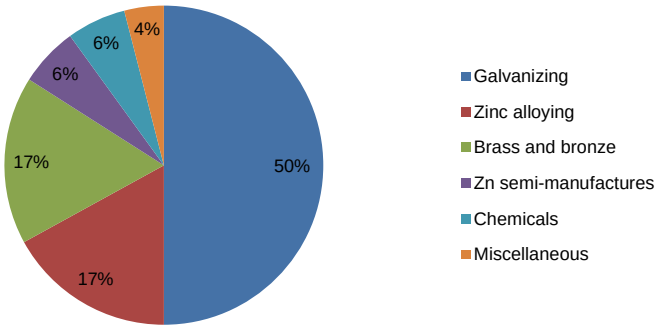

Figure 1.2 Primary use of Zn (ILZSG 2015)

1.2.4 Zinc in the aquatic environment

Zinc is the 25th most abundant element in the earth crust (Luoma & Rainbow 2008). Zinc in aquatic environments may originate from both natural as anthropogenic sources. Natural sources of Zn include natural erosion processes, and atmospheric deposition due to Zn emissions following volcano eruptions, forest fires, dust storms and sea sprays. Anthropogenic sources of Zn are corrosion of galvanized products and Zn alloys, industrial point sources, atmospheric deposition, wastewater treatment plants, road runoff and drainage from agricultural soils and sedimentation (Hogstrand 2012). Locally also discharges from local mining and metal-related industrial activities are of importance. Total concentrations in European rivers unaffected by historical mining or point sources have been reported to range between 5.4 and 42.6 µg/L (Van Sprang et al. 2009), although Zn concentrations near mining sites can be much higher (Luoma & Rainbow 2008).

1.2.5 Historical and current use of Pb

Lead ores have been extracted since the neolithicum, because of its co-occurrence with silver (Nriagu 1998). The production of Pb increased rapidly during the Roman Empire (50 000 tonnes per year; Nriagu 1998). This was due to the well-developed lead technology in the Roman Empire: lead was used in the production of pipes, aqueducts, food and drink containers, paints and was also used as a wine sweetener (Skerfving & Bergdahl 2007). Productions decreased again during the middle ages until the industrial revolution, when the demand for Pb raised again dramatically due to improvements in lead manufacturing technologies, and the increasing world population (Nriagu 1996; 1998). Lead production peaked again around the 1970s-1980s, amongst others due to the use of organic lead compounds as a fuel additive (Nriagu 1998). The global mine production of Pb was estimated in 2014 at 5 million tonnes per year, although the overall Pb consumption is around 11 million tonnes per year due to efficient recycling (ILZSG 2015). The most important Pb mining countries today are China, Australia and the United States (USGS 2013b).

The most important application of Pb nowadays is the production of lead-acid batteries (~80% of the Pb consumption) mainly used in vehicles (ILZSG 2015). Other current applications are the use in pigment in paints (5%), ammunitions (3%), rolled and extruded products (6%), alloys (2%), and cable sheathing (1%). An overview of the today's primary use of Pb is given in Figure 1.3.

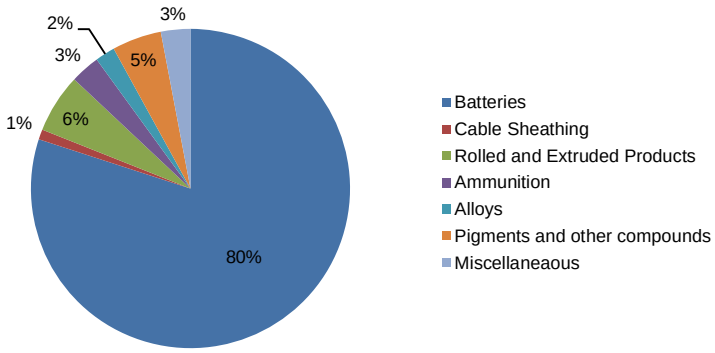

Figure 1.3. Primary use of Pb (ILZSG 2015)

1.2.6 Pb in the aquatic environment

Lead is the 36th most abundant elekement in the earth crust (Luoma & Rainbow 2008). Lead in aquatic environments may originate from both anthropogenic and natural sources. However, natural inputs are generally relatively low compared to Pb input originating from anthropogenic sources (Nriagu 1990). Emission to the environment may occur at all steps in the manufacturing process. The most important Pb input was the atmospheric deposition of Pb, mainly originating from the combustion of lead-containing gasoline. However, due to the phasing-out of leaded gasoline in Europe and North-America atmospheric deposition of Pb via this route has declined over the past decades (Nriagu 1996). Other important anthropogenic Pb inputs are originating from metal manufacturing, dumping of sewage sludge, domestic wastewater and smelting and refining of non-ferrous metals (Mager 2012). Dissolved Pb concentrations in European rivers unaffected by historical mining or point sources have been reported to range between <0.005 and 6.4 µg/L (FOREGS 2005), but locally concentrations can be much higher due to anthropogenic pollution.

1.2.7 Environmental implications

Metals are and have been indispensable in our society. Since the early start of metal mining, metals have been emitted to local ecosystems (Nriagu 1990). Estimates suggest that the anthropogenic inputs in metal biogeochemical cycles are as important as natural inputs (Rauch & Pacyna 2009). Anthropogenic pollution has led worldwide to elevated metal concentrations in aquatic ecosystems (Chau & Kulikovsky-Cordeiro 1995; Luoma & Rainbow 2008; Van Sprang et al. 2009; Stockdale 2010). The toxicological implications of these elevated concentrations endanger natural communities. This has urged authorities all over the world to develop Environmental Quality Standards (EQS) and risk assessment approaches, such as the Water Framework Directive (WFD) in Europe (EC 2001) and the Clean Water Act in the USA (USEPA 1987). The importance of taking into account metal bioavailability has been recognised by some regulatory authorities (e.g. EC 2003). In the last decade, European risk assessment procedures for metals, such as Zn and Ni have implemented bioavailability normalization based approaches (DEPA 2008; Van Sprang et al. 2009) and Ni and Pb EQS under the WFD are bioavailability based. However, metals mostly occur as mixtures in the environment, while most regulatory frameworks currently consider only the risks on a metal-by-metal basis. The incorporation of

metal mixture toxicity in these frameworks currently poses a new challenge for the regulatory authorities worldwide (Van Genderen et al. 2015; Meyer et al. 2015a).

1.3 Metal toxicity in daphnids

1.3.1 Essentiality versus non-essentiality

It has been long recognized that some metals are required in small doses to sustain life process. Essential metals play a biochemical role in the metabolic processes of organisms (Luoma & Rainbow 2008). The essentiality of a metal has been defined by: (I) its presence in living matter, (II) its ability to interact with living systems, and (III) the occurrence of deficiency symptoms when the metal is removed from the environment (Mertz 1974).

A typical example of an essential metal is Zn. Zinc is a cofactor in 10% of all proteins and plays as such a fundamental role in the metabolism of organisms, for instance as a cofactor in proteins such as the carbonic anhydrase enzyme or in important processes such as the immune system and cell signalling (Hogstrand 2012). Zinc has been shown to be essential for all known organisms (Vallee 1986). Deficiency symptoms for Zn have been reported for several aquatic species among which *Ceriodaphnia dubia* and *Daphnia magna* (Muyssen et al. 2001; 2002).

Although the essentiality of Ni to a variety of terrestrial organism, such as chickens, rats, pigs and cows, has been widely accepted (reviewed by Phipps et al. 2002), the essentiality of Ni in aquatic organisms has been far less well established (reviewed by Muyssen et al. 2004). For plants and marine algae Ni has been identified to be an essential cofactor for the urease enzyme involved in the nitrogen metabolism (Gordon et al. 1978; Rees & Bekheet 1982). For aquatic animals, no Ni-containing biomolecules have been identified (Muyssen et al. 2004). However, Ni is actively regulated in aquatic organisms (Muyssen et al. 2004; Chowdhury et al. 2008). Since the active regulation has not yet been observed for non-essential metals, it has been argued that Ni is also essential to aquatic animals (Muyssen et al. 2004; Chowdhury et al. 2008; Pyle & Couture 2012). However, this hypothesis has not yet been confirmed. Nickel deficiency to aquatic organisms has, to our knowledge, not yet been reported, although it has been suggested that the Ni requirements are probably rather low. Therefore, Ni deficiency would be a quite rare phenomenon in natural environments (Pyle & Couture 2012).

Lead, on the contrary, is a non-essential metal. There is no evidence of a biological function for Pb and as such Pb is considered to be not required nor beneficial for life (Mager 2012).

1.3.2 Mechanisms of chronic metal toxicity

All metals can be toxic, whether they are essential or not. Toxic effects occur once a certain threshold of metal availability is reached (Luoma & Rainbow 2008). Toxicity in aquatic organisms is actually the endpoint of a complex process involving several metal-organism interactions. First, the metal has to diffuse through the protective layers surrounding the biological surface (e.g. mucus in the case of animal cells). Second, the metal is transported through the eukaryotic lipid bilayer facilitated by passive transporting proteins, either carriers or channels. Once inside the cell, the metal is considered to be metabolically active, i.e. it has the potential to bind with molecules in the cell. The essential metal can bind to the ligands of the biomolecules for which it is involved in the biological function, e.g. Zn in the carbonic anhydrase enzyme. Alternatively, the non-essential metal or when in excess also the essential metal has to be detoxified. Several cellular processes exist to detoxify excess metals, such as sequestering in granules and binding to methallothioneins. Finally, the metal can be excreted (Campbell 1995; Luoma & Rainbow 2008). Metal toxicity occurs when metabolically active metal accumulates at sites of toxic action due to an imbalance of the uptake and detoxification and excretion processes (Luoma & Rainbow 2008). Metal uptake may happen either at the interface of water-organism (waterborne toxicity) or of the gut lumen-organism (dietary toxicity). The present study focusses on waterborne toxicity.

Generally, the disruption of the ion homeostasis is considered as one of the most important mechanisms of metal toxicity (Paquin et al. 2002) The mechanism of metal toxicity in daphnids is not that well understood, but will be discussed below.

1.3.3 Mechanisms of Ni toxicity

Chronic Ni toxicity has been shown to influence daphnid reproduction and survival (Biesinger & Christensen 1972; Müzinger 1990; Pane et al. 2004; De Schamphelaere et al. 2006; Deleebeeck et al. 2007; 2008), growth (Biesinger & Christensen 1972; Müzinger 1990; Enserink et al. 1991;

Pane et al. 2004), intrinsic rate of increase (Müzinger 1990; Enserink et al. 1991), first brood size (Müzinger 1990), and population yield (Enserink et al. 1991).

Pane et al. (2003a) investigated the mechanisms of acute and chronic toxicity to *D. magna*. These authors observed that whole body Mg concentrations and Mg^{2+} uptake were significantly reduced during acute and chronic exposure to Ni. The latter suggests that Ni most probably works as a Mg antagonist at Mg^{2+} uptake sites, which agrees with the observations of the protective effect of Mg on chronic Ni toxicity to daphnids (Deleebeeck et al. 2008). Nickel exposure had also minor effects on Na^+ and Cl^- balances, while it did not affect the Ca^{2+} balance. In fish, acute Ni toxicity has been linked to a disruption of the respiratory mechanisms rather than of the ionoregulatory mechanisms (Pane et al. 2003b). For *D. magna,* a significant decrease in the oxygen consumption rate and whole body haemoglobin concentrations was observed after chronic exposure (Pane et al. 2003a). This all suggests that the mechanisms of chronic Ni toxicity are both ionoregulatory and respiratory.

1.3.4 Mechanisms of Zn toxicity

Chronic Zn toxicity has been shown to influence daphnid reproduction (Belanger & Cherry 1990; Masters et al. 1991; Heijerick et al. 2005; De Schamphelaere et al. 2005; Muyssen et al. 2006; Cooper et al. 2010), survival (Enserink et al. 1991; Masters et al. 1991; Muyssen et al. 2006; Cooper et al. 2010), growth (Enserink et al. 1991; Muyssen et al. 2006), intrinsic rate of increase (Enserink et al. 1991; Sanchez-Ortiz et al. 2010), and population yield (Enserink et al. 1991; Sanchez-Ortiz et al. 2010).

For fish the primary mode of action of acute Zn toxicity is the inhibition of the Ca^{2+} uptake, which leads to hypocalcaemia (Spry & Wood 1985, Hogstrand et al. 1995). However, during chronic exposure to sublethal concentrations calcium balances in fish are restored due to acclimation effects (Hogstrand et al. 1995). Muyssen et al. (2006) investigated the effects of chronic Zn exposure on mortality, growth, reproduction, filtration and ingestion rate, respiration rate, energy reserves, internal Zn and Ca concentrations in *D. magna*. Mortality occurred mainly in the first week of exposure and was associated with a decrease of the Ca content in the daphnids. This indicates that short-time toxicity to daphnids may also occur via an inhibition of

the Ca uptake. The hypothesis that Zn acts as a Ca antagonist is corroborated with the observation of the protective effect of Ca on acute and chronic Zn toxicity (Heijerick et al. 2003; 2005). Zinc exposure did also decrease filtration rate, weight and the overall energy reserves (Muyssen et al. 2006). However, during the following two weeks general repair possesses were clearly active since Ca uptake, filtration rate as well as energy reserves were restored. This all suggest that, similar as for fish, chronic effects of Zn observed for daphnids are mainly caused by the short-term effects on the Ca homeostasis.

1.3.5 Mechanisms of Pb toxicity

Chronic Pb toxicity has been shown to affect daphnid reproduction (Cooper et al. 2010; Mager et al. 2011a; Esbaugh et al. 2012), survival (Enserink et al. 1991; Cooper et al. 2010; Mager et al. 2011a; Esbaugh et al. 2012), growth (Enserink et al. 1991), intrinsic rate of increase (Enserink et al. 1991), and population yield (Enserink et al. 1991).

Inhibition of the Ca^{2+} uptake, which results in hypocalcaemia, has been identified as the primary mechanism of acute Pb toxicity in fish, although Na^{+} and Cl^{-} uptake are also affected (Rogers et al. 2003; Rogers & Wood 2004). Ca has been shown to protect against both acute and chronic toxicity to fish (Grosell et al. 2006a; Mager et al. 2011b), which suggests that Pb indeed works as a Ca antagonist. However, similar as for Zn, Ca levels in fish are restored after prolonged exposure, and mortality effects observed during chronic exposure are mainly caused by the effects during the early period of the exposure (Grosell et al. 2006a). We are not aware of any studies investigating the mechanisms of chronic Pb toxicity to daphnids. While Ca has been shown to protect against acute toxicity to *C. dubia* (Mager et al. 2011b), the effects of Ca on chronic toxicity have not yet been unambiguously established. Mager et al. (2011a) did not observe protective effects of Ca on chronic Pb toxicity. Parametrix (2010), in contrast, reported a decreased chronic Pb toxicity with increasing ambient Ca concentrations, although the results may have been confounded by correlated increases in pH and alkalinity. However, a recent study reported that the disturbance of the Ca homeostasis is not likely to be the primary mechanism of chronic Pb toxicity to the freshwater snail, *Lymnaea stagnalis* (Brix et al. 2012).

1.4 Effects of water chemistry on metal toxicity

The importance of the effects of water chemistry on metal toxicity has long been recognized (Campbell 1995; Paquin et al. 2002). Water chemistry can affect metal toxicity via two different mechanisms. First, speciation interactions occurring in the solution may affect metal toxicity. Second, certain ions in the solution may compete with metals for binding at uptake sites or the sites of toxic action. As a consequence neither total nor dissolved concentrations have been shown to be good predictors of metal toxicity.

1.4.1 Effect of speciation on metal toxicity

Speciation has been defined as "the distribution of an element amongst defined chemical species in a system" (Templeton et al. 2000; Nordberg et al. 2009). In chemical terms, species in turn can be defined as 'the specific form of an element defined as the isotopic composition, electronic or oxidation state, and (or) complex or molecular structure" (Nordberg et al. 2009). In freshwater, metals can occur as a variety of species. Speciation is of main importance for metal toxicity, since not all metal species are bioavailable, i.e. not all metal species are taken up by an organism from the environmental medium (Luoma & Rainbow 2008). Speciation may act at two levels. First, metals either associate with the particulate matter in suspension, or are present in the dissolved fraction. Particulate metals may contribute to toxicity via dietary uptake (e.g. Evens et al. 2009). Although dietary metal uptake is for some metals the most important exposure pathway for metal toxicity, for most metals the importance of the dietborne route for metal toxicity is relatively minor compared to the waterborne route (DeForest & Meyer 2015).

Second, in the dissolved fraction, metals may be present as free metal ion, often denoted as Me^{2+}, or may be complexed to either organic or inorganic ligands. The free metal ion (Me^{2+}) is generally considered as the metal species which is the most bioavailable and thus most toxic species (Campbell 1995; Paquin et al. 2002). However, several exceptions to this rule have been reported (see Campbell 1995 for an overview) and inorganic and organic ligand-metal complexes may also be bioavailable.

The relative distribution of the metal species is dependent on the water chemistry. Low pH generally increases the free metal ion concentration due to the competition between protons

and metal ions for complexation with organic ligands. On the other hand, the presence of inorganic (e.g. Cl^-, SO_4^{2-}, OH^-, HCO_3^- and CO_3^{2-}) and organic ligands (dissolved organic matter (DOM), e.g. fulvic and humic acid) will promote the complexation of metal ions, and thus decrease toxicity. In many natural waters, DOM is the most important parameter determining metal speciation. It is generally accepted that DOM has a protective effect on metal toxicity through its ability to complex metals (Wood et al. 2011).

For most metals, the chemical speciation in solutions is difficult to measure. Alternatively, chemical speciation can be calculated using computational models. The Windermere Humic Aqueous Model VII (WHAM VII; Tipping et al. 2011) and Visual Minteq 3 (Gustafson 2014) are equilibrium chemical speciation model for surface and soil waters. These models calculate chemical speciation of the metal ion and inorganic complexes based on an extensive thermodynamic database containing stability constants for inorganic complexes. In the present study, both speciation models have been used to calculate metal speciation.

Both speciation models also include a model describing the interactions of metals at the binding sites of NOM. In WHAM VII, the Humic Ion-Binding Model VII is used to model binding of metals and protons to humic and fulvic acids (Tipping et al. 2011). The Humic Ion-Binding Model VII calculates NOM-metal interactions based on a set of intrinsic equilibrium constants. Metals are assumed to bind at bidentate and tridentate metal binding sites taking into account heterogeneity in binding strength. Accumulation of the metals in the diffuse layers surrounding the organic molecules is calculated using a non-specific electrostatic binding model, the Donnan model. Model VII replaces the previous versions of the Humic Ion-Binding Model (Model V and Model VI). WHAM VII has been shown to be able to relatively accurately reproduce field measurements of free metal ion concentrations for several metals, such as Ni, Zn and Pb, at concentrations relevant for metal toxicity (Lofts & Tipping 2011). Additionally, it has recently been used to model metal-NOM interactions in several mixture studies (Tipping & Lofts 2013; 2015; Iwasaki & Brinkman 2015).

Visual Minteq (Gustafson 2014) is a freeware chemical equilibrium model that calculates metal speciation using thermodynamic databases with the stability constants for inorganic complexes reported by the National Institute of Standards and Technology (NIST). In the present study, metal-NOM interactions in Visual Minteq were modelled using the Non-Ideal Competitive

Adsorption (NICA)-Donnan model (Milne et al. 2001; 2003). The NICA-Donnan model is a multiple-site model that can account for non-ideal binding of metals to heterogeneous organic ligands and non-specific electrostatic binding model around the organic material (Koopal et al. 1994; Benedetti et al. 1995; Kinniburgh et al. 1996). Visual Minteq has been used to calculate Pb speciation in several recent studies (Esbaugh et al. 2012; De Schamphelaere et al. 2014) since is the only available speciation software that allows in a single framework the calculation of formation of inorganic Pb complexes, complexes of Pb with humic acid (HA) and fulvic acid (FA), and precipitation of minerals (e.g. $Pb(OH)_2(s)$, cerrusite, and hydrocerrusite).

1.4.2 Competitive effects at uptake sites

The idea that hardness cations (Ca^{2+} and Mg^{2+}) can compete with metals for binding at 'active sites' has first been raised by Zitko and Carson (1976). These authors suggested that Mg^{2+} cations may compete with Zn^{2+} for binding at sites of action in fish tissue and as such Mg protects against Zn toxicity. A decade later, researchers learned that protons affect metal toxicity not only by their effect on speciation, but also by competitive interactions at the biological surfaces (Campbell & Stokes 1985; Cusimano et al. 1986). Today, the protective effects of certain cations, such as H^+, Ca^{2+}, Mg^{2+} and Na^+, on metal toxicity are generally accepted. However, which cation protects against toxicity is dependent on the metal, the organism considered and the duration of exposure (i.e. acute vs. chronic exposure).

Heijerick et al. (2005) explained the observed protective effects of Ca^{2+}, Mg^{2+}, Na^+ and H^+ on chronic Zn^{2+} toxicity to *D. magna* as competition effects of these cations at the site of action. Protection against chronic Ni^{2+} toxicity to *D. magna* is afforded by Mg^{2+}, Ca^{2+} and H^+ (Deleebeeck et al. 2008). The protective effects of Mg were explained as a competition effect with Ni^{2+} for uptake at the Mg^{2+} transport sites. The effect of Ca^{2+} on Ni^{2+} toxicity, on the other hand, was attributed to secondary effects of Ca in the maintenance of the cell membrane integrity. The effects of H^+ were partly explained by competitive effects between protons and Ni for uptake, although also other unidentified effects most likely contributed. The protective effects of cations on chronic Pb toxicity to daphnids have not yet been fully unraveled. As mentioned above, the effects of Ca on Pb toxicity to daphnids were at the start of this study still unclear. Mg^{2+} and Na^+, on the other hand, were shown not to affect chronic Pb toxicity to *C. dubia* (Mager et al.

2011a). Protons protect against chronic Pb toxicity, although the mechanism; either competition at the uptake site or an effect on physiology, remains unclear (Esbaugh et al. 2012).

1.4.2 Modeling metal bioavailability and toxicity

After the first observation of the effects of metal speciation and competition influencing metal toxicity, scientists have tried to rationalize these experimental observations. This led to the formulation of two bioavailability models: the free ion activity model (FIAM) by Morel (1983) and the gill surface interaction model (GSIM) by Pagenkopf (1983). Although there are some differences, both models assume that the toxic response is the consequence of a metal species binding at the site of toxic action. These early bioavailability models based on chemical equilibration formulation considered also the competition between the bioavailable metal species and cations for binding at the cellular sites. At the time the FIAM and GSIM were presented, they were not really accepted as useful tools for deriving EQS in regulatory framework, although they share many characteristics of the bioavailability models that are now generally accepted by regulatory authorities. In the following decades, the increased understanding of the physiological mechanisms of metal toxicity (reviewed by Paquin et al. 2002) eventually led to the formulation of the biotic ligand model (BLM) of acute toxicity around the turn of the millennium by Di Toro et al. (2001).

The BLM assumes that the metal ions bind at a biotic ligand site (Di Toro et al. 2001). For fish, this biotic ligand site is a specific site at the surface membrane of the gills, the primary sites of toxic action for fish. For other organisms, it is hypothesized that metal ions bind at a conceptual biotic ligand site. A toxic response occurs when a critical concentration of metal binding at the biotic ligand site is reached. For instance, the LA50 is the lethal accumulation at 50% mortality and thus represents the organism's acute sensitivity. The LA50 is assumed to be constant irrespective of the water chemistry (Meyer et al. 1999). The amount of metal binding at the biotic ligand site is dependent on the concentration of bioavailable metal species and the concentrations of competitive cations, e.g. Ca^{2+}, Mg^{2+}, H^{+}, Na^{+}, in the solution. For fish, the fraction of the metal binding at the gills is predicted using conditional binding stability constants for both metal ions and of the competing cations, which are derived from direct measurements of gill tissue (e.g. Playle et al. 1993; Janes & Playle 1995). However, for small animals, such as

daphnids, measurements of gill accumulation concentrations are too difficult. Therefore, in the framework of Di Toro et al. (2001) it is assumed that binding stability constants are the same for all organisms, while the LA50 is organism-specific (e.g. Santore et al. 2001).

Around the same time, an alternative modelling approach was presented by De Schamphelaere and Janssen (2002). In this approach, the basic assumptions about binding of the metal to a biotic ligand and the competitive effects of cations are the same as for the BLM approach of Di Toro et al. (2001). However, the approach of De Schamphelaere & Janssen (2002) allows that biotic ligand binding stability constants are calculated directly from the observed toxicity data. The method requires the assessment of the univariate effects of possible competing anions on metal toxicity (see Chapter 2). Biotic ligand stability constants are derived from the linear relationship between the toxicity expressed as free metal activity (mostly $EC50_{Me2+}$) and the activity of the considered competing cation. The model formulation for the situation where Ca^{2+}, Mg^{2+}, Na^{+}, and H^{+} compete with metal Me^{2+} for binding at the biotic ligand sites is given by Equation 1.1 (De Schamphelaere & Janssen 2002).

$$EC50_{Me^{2+},pred} = \frac{f_{MeBL}^{50\%}}{(1-f_{MeBL}^{50\%})\cdot K_{MeBL}}\left(1 + K_{CaBL}\{Ca^{2+}\} + K_{MgBL}\{Mg^{2+}\} + K_{NaBL}\{Na^{+}\} + K_{HBL}\{H^{+}\}\right) \qquad (1.1)$$

In Equation 1.1, $EC50_{Me2+,pred}$ is the predicted 50% effective concentration of metal Me^{2+} (mol/L). $f_{MeBL}^{50\%}$ is the fraction of available biotic ligand sites that are occupied by metal Me^{2+} at the 50% effect level and is independent of the water chemistry (De Schamphelaere & Janssen 2002). K_{MeBL}, K_{CaBL}, K_{MgBL}, K_{NaBL} and K_{HBL} are the biotic ligand stability constants for metal Me^{2+}, Ca^{2+}, Mg^{2+}, Na^{+} and H^{+} (in L/mol), respectively. $\{Ca^{2+}\}$, $\{Mg^{2+}\}$, $\{Na^{+}\}$, and $\{H^{+}\}$ are the activities of Ca, Mg, Na, and protons in the solution (in mol/L), respectively.

The application of the latter BLM modelling approach was first investigated for the acute toxicity of Cu to *D. magna* (De Schamphelaere & Janssen 2002). Meanwhile, it has also been successfully used to predict acute and chronic toxicity of Cu and Zn to several aquatic organisms, such as *D. magna* (De Schamphelaere & Janssen 2004a; De Schamphelaere et al. 2005; Heijerick et al. 2005).

De Schamphelaere & Janssen (2002) implicitly assumed in their approach that the relationship between free metal toxicity and each of the competing cations is linear. However, the relationship between metal toxicity and proton activity is not always linear, but can be rather

curvilinear (e.g. De Schamphelaere & Janssen 2002; Heijerick et al. 2005; Deleebeeck et al. 2007; 2008; 2009; Esbaugh et al. 2012). This suggest that also other factors besides the competitive effect of H^+ may be important in determining the effect of pH on free metal toxicity. As a consequence, an alternative model formulation was adopted in the chronic Ni bioavailability models for *C. dubia, D. magna,* fish and algae (De Schamphelaere et al. 2006; Deleebeeck et al. 2007; 2008; 2009). In these models the effect of H^+ on Ni^{2+} was expressed as a log-linear effect of pH (S_{pH}), which was superimposed on the traditional BLM-type linear effects of the other competitive ions (Equation 1.2 for the daphnid bioavailability models of De Schamphelaere et al. (2006) and Deleebeeck et al. (2008)).

$$EC50_{Ni^{2+},pred} = 10^{-(Q_{50}+pH \cdot S_{pH})}\left(1 + K_{CaBL}\{Ca^{2+}\} + K_{MgBL}\{Mg^{2+}\}\right) \quad (1.2)$$

In Equation 1.2, $EC50_{Ni2+,pred}$ is the 50% effective concentration of Ni^{2+}, expressed as free ion activity (mol/L). Q_{50} is the intrinsic sensitivity (i.e. the intercept of the linear relationship between pH and the negative logarithm of the hardness corrected observed $EC50_{Ni2+}$; Deleebeeck et al. 2008; unit: log(mol/L)). This log-linear model structure deviates from the original BLM approach and therefore the more general term bioavailability model instead of BLM is preferred for these type of models.

Irrespective of which model formulation is used, essentially al bioavailability model are similar in structure (see Figure 1.4 for a schematic overview). Metal bioavailability models typically contain three components: speciation, competition, and (intrinsic) sensitivity. As described above the speciation determines the bioavailability of the metal: metals in the solution can be complexed to either organic (e.g. DOC) or inorganic (e.g. hydroxides, chlorides, carbonates, sulfates) ligands. The complexation with ligands lowers in most cases the bioavailability of the metal, since the metal complexes are usually not considered to be bioavailable to organisms, although exceptions exist (Campbell 1995). The speciation component of the bioavailability model is usually modelled using speciation software. Currently, different speciation software programs are used for different metals, e.g. WHAM V for Zn, WHAM VI for Ni, and Visual Minteq for Pb. The latter is a primarily consequence of the historical sequence of the development of bioavailability models. In the early 2000's, biotic ligand models were developed for Zn, and also Cu. At that time WHAM V was considered to be the most appropriate speciation program. However, at the time that the Ni bioavailability models were developed WHAM V was replaced by WHAM VI, which described the ion-binding with humic substances more accurately than its predecessor. For

Pb, on the other hand, Visual Minteq is used as speciation software, since it is the only software that can calculate both Pb^{2+} activities, Pb precipitation and (in)organic Pb complexation.

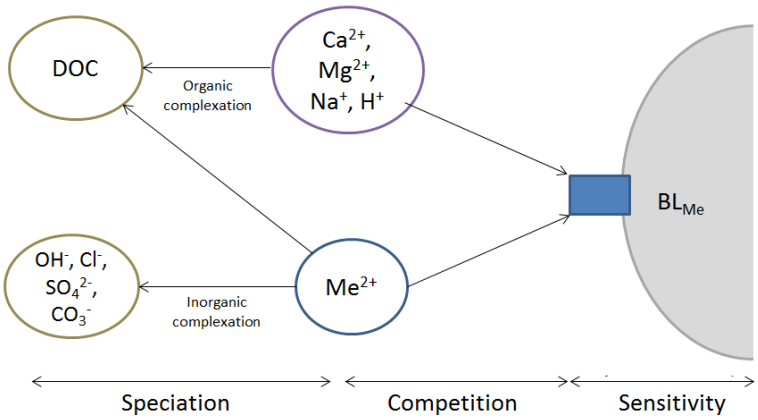

Figure 1.4. General schematic representation of metal bioavailability models.

The competition component considers the binding of the free metal ions in the solution to the biotic ligand site (BL_{Me}) including the possible competitive effects of major cations (such as Ca^{2+}, Mg^{2+}, Na^{+} and H^{+}) for binding at the biotic ligand sites. An example of the competition component of a bioavailability model is given in Equation 1.1 and 1.2. The sensitivity component is determined by the physiological interactions occurring in the organism. The sensitivity is described by the intrinsic sensitivity parameter in the bioavailability model (e.g. $f_{MeBM}^{50\%}$ and Q50 for Equation 1.1 and 1.2, respectively), and is a measure for the amount of metal bound to the biotic ligand.

1.5 Mixture toxicity

Most current environmental risks assessment procedures are still based on a substance-by-substance approach, although organisms in the aquatic environment are usually exposed to mixtures of substances. The observation that the effect of a mixture may differ extensively from the effects of the individual constituents in the mixture has challenged scientist with the question how to assess mixture toxicity effects (Altenburger et al. 2013). Quite quickly, it was

accepted that testing of every possible mixture combination occurring in the environment is unrealistic. Instead, efforts have been directed to investigate whether mixture toxicity can be predicted based on the individual toxicity of each of the mixture constituents.

1.5.1 Mixture reference model

Currently, two mixture reference models are generally accepted: the concentration addition (CA) and independent action (IA) model (Jonker et al. 2005; Jonker et al. 2011). Both models assess mixture effects based on the effects of the individual mixture constituents. One of the most important differences between these mixture reference model are the assumptions about the underlying mechanisms of how the mixture constituents affect organisms.

The CA model was originally described by Loewe and Muischneck (1926). The concept can be mathematically expressed using Equation 1.3.

$$\sum_{i=1}^{n} TU_i = \sum_{i=1}^{n} \frac{c_i}{ECx_i} = 1 \qquad (1.3)$$

Where n is the number of mixture constituents, TU_i is the toxic unit of the i^{th} mixture component. The TU_i is defined as the ratio between c_i, the concentration of the i^{th} mixture component, and ECx_i, the x% effective concentration of the i^{th} mixture component (when applied singly). If the CA model holds, then the sum of toxic units (∑TU) equals 1 in a mixture causing x% effect. The CA model assumes that substances have the same mode of action. Additionally, it assumes that a substance in a mixture can be exchanged for other substances without changing the overall mixture toxicity, as long as the sum of toxic units of the mixture does not change.

Alternatively, the IA model assumes that substances have a different mode of action. The IA model is based on the concept of independent random events (Bliss 1939). The IA assumes that the joint response to a mixture (y_{mix}) can be calculated as the product of the responses to each of the individual components in the mixture (y_i) (Equation 1.4), but is often represented with the equivalent Equation 1.5.

$$y_{mix} = \prod_{i=1}^{n} y_i \qquad (1.4)$$

$$E_{mix} = 1 - (\prod_{i=1}^{n}(1 - E_i)) \quad (1.5)$$

In Equation 1.5, E_{mix} is the proportional joint effect of the mixture, and E_i is the proportional individual effect of the i^{th} mixture component if applied singly.

The CA and IA model fundamentally differ in how they assess effects of mixtures wherein the constituents are present at low concentrations. Based on Equation 1.4, the IA model assumes for a mixture where each of the mixture constituents is present at a concentration that does not cause effect, that there would also be no mixture effect. Only components that are present at a concentration that causes an effect (i.e. E_i>0) will actually contribute to the joint effect. According to the CA model, all mixture components contribute to the overall mixture toxicity, proportional to its toxic unit. This implicates that the joint effect of a mixture wherein the mixture components are present at low concentrations, for example all at their EC1 (i.e. the 1% effective concentration) is dependent on the number of mixture components.

When mixture effects are evaluated using the above described reference models, it is essential that the toxicity of the mixtures are investigated simultaneously with those of each of the individual components in the mixture. De Laender et al. (2009) demonstrated that when the toxicity is not-simultaneously assessed erroneously conclusions about the interactive effects can be made.

1.5.2 Interactive effects

Both reference models (CA and IA) depart from the idea of non-interactivity, i.e. substances do not interact, also described as 'additivity'. However, this assumptions is not always fulfilled and substances often do interact when combined in a mixture. An interaction is believed to occur when a component influences the amount of another component accumulating at target site or his activity at that target site (Jonker et al. 2011). This interaction at the target site ultimately results in a different response than what can be expected based on the individual components. As a consequence, the interpretation of a mixture effect is dependent on the considered mixture reference model.

If the observed mixture effect is larger than expected based on the reference model, the mixture acts synergistically (also 'more than additive' is used). In contrast, antagonistic interactions occur if the observed mixture effects are smaller than those predicted with these models (also 'less than additive' is used) (Jonker et al. 2005; 2011).

1.5.3 Mixture test designs

Several test-designs have been used to investigate mixture toxicity: e.g. ray design; full-factorial designs, fractional factorial designs, isoboles (Jonker et al. 2011). The two designs used in the present study will be briefly addressed below.

The full-factorial design investigates mixture toxicity over the complete concentration-response surface (Jonker et al. 2011; Figure 1.5.A). In the full-factorial design all single substance treatments are also combined in mixture treatments. The design is used to screen for systematic deviations from non-interactivity, such as deviations that are concentration level dependent or effect size dependent (e.g. Chapter 5). The design is mostly used to investigate binary mixtures, because the number of test combinations increases exponentially with the number of mixture components.

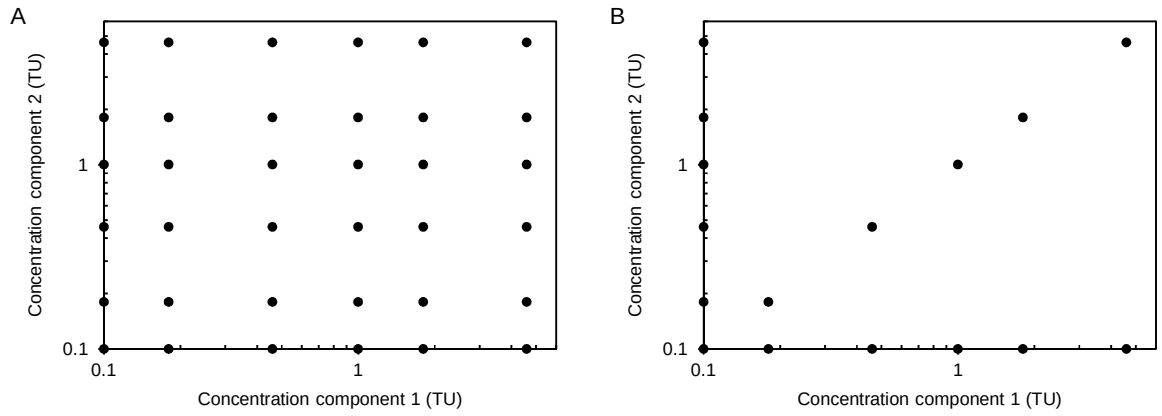

Figure 1.5. Example of the full-factorial (A) and ray-design (B; the quitoxic ray) for binary mixtures.

The ray design, or 'fixed ratio design', investigates toxicity in a mixture wherein the concentration ratio of the components is kept constant, but the total concentration of the mixture is varied (Jonker et al. 2011; Figure 1.5.B). The design generates a concentration response curve for a mixture, which can be analysed in a similar way as the concentration response curves of the individual substances. The often used equitoxic ray is a ray in which components are combined at the same toxic strength (i.e. same TU per component). By investigating several rays differing in the concentration ratios, concentration-ratio-dependent interactions may also be identified.

1.5.4 Metal mixture toxicity

Although the toxicity of metal mixtures has been investigated for decades, no clear patterns have emerged from these studies. Two meta-analysis evaluated the mixture effects observed in metal mixture studies (Norwood et al. 2003; Vijver et al. 2011). Both authors found that interactive effects (i.e. antagonisms and synergism) were more commonly observed than non-interactive effects. Furthermore, the observed interactive effects were highly variable and may depend on the test organism, metal combination, metal concentration ratio, metal concentrations, water chemistry, exposed life stage, exposure duration and considered endpoint.

The picture is further complicated by the paucity of chronic metal mixture toxicity data. The majority of studies reported metal mixture effects during acute exposure (Meyer et al. 2015a), while regulatory frameworks such as the WFD in Europe mostly rely on chronic toxicity data. Furthermore, it has been noted that a substantial number of metal mixture studies may have reported data obtained using non-simultaneously toxicity testing of the individual metals and the metal mixture (Meyer et al. 2015a).

Interactions between metals may occur at the different levels involved in the toxicokinetic process. Additionally, since metals compete with each other for DOC binding sites, interactive effects may occur at the level of speciation. As a consequence, synergistic interactions at the dissolved level, may be actually non-interactive when expressed as the bioavailable free metal ion (Meyer et al. 2015b). Alternatively, metals may compete at the transport sites of the cell membrane, thereby influencing each other's uptake. For instance, the uptake of Ni by *D. magna* has been shown to be suppressed in the presence of Zn, which suggest that competitive effects

at the uptake sites may take place (Komjarova & Blust 2008). Once inside the organism, metals may affect each other's toxicification and detoxification pathways by binding at target proteins. For instance, metallothionein concentrations in the midgut of shore crabs exposed to a mixture of Cd-Zn was substantially larger than the concentrations in crabs exposed to either Cd or Zn individually (Martín-Díaz et al. 2005). Since metal ions often show chemical and physical similarities, non-essential metals may bind to the binding site of essential metals in proteins and as such change the biochemical function of these proteins. It is the combination of all these processes that eventually leads to the mixture effects observed at the toxicity level. Therefore, interactive mixture effects observed at the physiological level do not necessarily reflect toxicity effects (e.g. Sharma et al. 1999).

1.5.5 Metal mixture bioavailability models

Di Toro et al. (2001) mentioned that the BLM-concept can also theoretically be used to predict the toxicity of metal mixtures. Playle (2004) was the first who actually explored the use of a metal mixture bioavailability model in explaining metal mixture toxicity using simulations. Since then several studies have used BLM-type models to predict metal mixture toxicity (e.g., Hatano & Shoji 2008; Kamo & Nagai 2008; Jho et al. 2011). These early mixture bioavailability models assumed that metals bind at a single shared biotic ligand site and are therefore CA-based. However, more recently IA-based BLMs that assume multiple biotic ligand sites have been presented (e.g. Versieren et al. 2014; Santore & Ryan 2015).

Of particular importance in this regard, is the recent metal mixture modelling evaluation in which four metal mixture bioavailability models were intensely studied and tested on a variety of metal mixture toxicity datasets (e.g. Van Genderen et al. 2015; Farley et al. 2015). All of these models were similar in structure since they all accounted for geochemical speciation effects and biological interactions (i.e. competitive binding and metal accumulation). However, they differed in their underlying assumption, technical basis and calibration strategy (Van Genderen et al. 2015; Farley et al. 2015).

Three of the four evaluated metal mixture bioavailability models were based on the BLM-concept. However, they differed in whether they assumed the existence of one type of biotic

ligand for all metals (Kamo & Nagai 2008; USGS model in Farley et al. 2015; Ywasaki et al. 2015) or multiple-biotic ligand sites (Santore & Ryan 2015), which consequently divides these models in CA-based or IA-based approaches, respectively. Furthermore, the models had different assumptions on the potency of the metals in causing toxicity. In the model of Kamo & Nagai (2008; see also Ywasaki et al. 2015) all metals have an equal potency, while the other models allow different metal potencies.

An alternative metal mixture bioavailability model is the WHAM F_{TOX} model, in which the binding of metals ions and protons on humic acid molecules calculated using the WHAM speciation software is used as a surrogate for competition and metal binding at the cell surface of aquatic organisms (Tipping & Lofts 2013; 2015). Every metal is considered to have a different potency in eliciting toxicity.

Based on the metal mixture modelling evaluation project it was concluded that the IA model is generally a better approach to model metal mixture toxicity. Additionally, although simple single-site models may be sufficient for many metal mixtures, models considering multiple binding sites may address antagonistic interactions better. Finally, it was shown that reasonable predictions of metal mixture toxicity can be achieved by calibrating the bioavailability models on the individual exposures (Van Genderen et al. 2015; Farley et al. 2015; Farley & Meyer 2015).

However, the metal mixture bioavailability models were mostly evaluated based on toxicity data from acute exposures, while the application for chronic datasets remains to be further evaluated (Van Genderen et al. 2015).

1.6 European metal regulation

1.6.1 Environmental Quality Standards

Several metals, i.e. Ni, Pb, Cd and Hg, have been identified as priority substances in the European WFD in 2001 (EC 2001). For these metals, EU-wide EQS were set in 2008 for which member states had to ensure compliance in all surface waters by 2015, e.g. EQS_{Ni} 20 µg dissolved Ni/L and EQS_{Pb} 7.2 µg dissolved Pb/L (based on the annual average; EC 2008). In 2013, EQS of priority substances were revised and following 'generic' (worst-case) EQS were adopted for Ni and Pb: EQS_{Ni} 4 µg dissolved Ni/L and EQS_{Pb} 1.2 µg dissolved Pb/L (EC 2013a).

Compliance to the EQS is demanded by 2021. However, the effects of water chemistry in determining metal toxicity was recognized and a bioavailability-based risk evaluation was allowed: *"Member States may, when assessing the monitoring results against the relevant EQS, take into account [...] hardness, pH, dissolved organic carbon or other water quality parameters that affect the bioavailability of metals"* (EC 2013a). Additionally, since certain metals may be naturally present in surface waters, natural background concentrations may be taken into account in determining EQS. A bioavailability based EQS may be used if bioavailability models (BLMs, regression, speciation) exist (EC 2011). Guidelines on how to determine bioavailability based EQS are given in the 'Technical guidance document for deriving environmental quality standards' (EC 2011). In short, a tiered approach is proposed: In Tier 1, compliance to the generic EQS as reported in the WFD (EC 2013a) is evaluated. If the measured concentration in the surface water is higher than this generic EQS a bioavailability correction may be considered. Two approaches are allowed: I) using a 'bioavailability factor' on the monitoring data (thus exposure data) or II) applying a bioavailability normalisation on the EQS to calculate a site-specific EQS.

Zn is currently not a priority substance in the European WFD. Member states are therefore responsible for setting EQS_{Zn}. In Flanders, for instance, a surface water EQS_{Zn} of 20 µg dissolved Zn/L has been adopted (VR 2015).

1.6.2 Risk Assessments

REACH, Registration, Evaluation and Authorization of Chemicals, in practice since 2007 is the current back-bone of chemical legislation in Europe (EC 2006). One of its goals is to protect human health and the environment from the risks that are associated with chemicals. It places the responsibility for assessing and managing risks with the industry. REACH replaces a number of different regulations and directives, such as the Existing Substances Regulation (EC 1993), which were in force before 2007. The REACH legislation requires that for all chemical substances manufactured and/or imported in the EU in quantities of more than 10 tonnes per year a chemical safety report should be composed. This chemical safety report should include a chemical safety assessment in which the risks of the substance to human health and the environment are assessed over the entire life-cycle. The methods for the chemical safety assessment are largely based on the requirements from the former European legislation

concerning chemical substances (EC 2003). The chemical safety assessment contains three separate steps: exposure assessment, effect assessment and risk characterisation. For metals, a bioavailability based effects assessment is allowed (ECHA 2008). Risk characterisation is performed by comparing the Predicted Environmental Concentration (PEC) with the Predicted No-Effect Concentration using the Risk Characterisation Ratio (RCR=PEC/PNEC). If the RCR is larger than 1, the substance is likely to pose risks to the community of the considered environmental compartment.

Bioavailability based PNECs have been used in European risk assessments, under the current or former chemical legislation, for metals such as Ni (DEPI 2008), Cu (ECI 2008), and Zn (Van Sprang et al. 2009). In these effect assessment approaches, bioavailability models, used to normalize chronic metal toxicity data (10% effective concentrations [EC10] or no-observable effect concentrations [NOEC]), are combined with species sensitivity distributions (SSD) to calculate 5% hazardous concentrations (HC5) (Figure 1.6 for Ni). The bioavailability corrected PNEC is derived from the HC5 by applying an assessment factor between 1 and 5 depending on the uncertainties associated with the methodology.

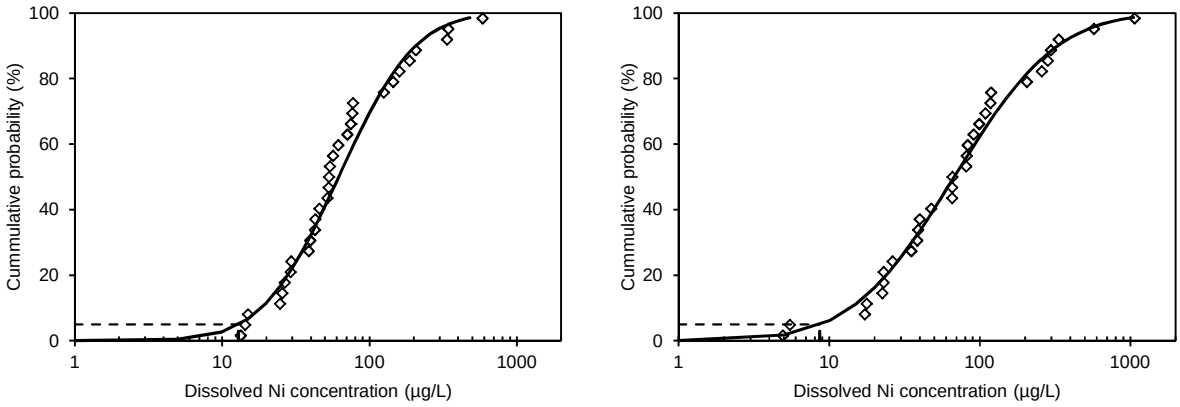

Figure 1.6. Bioavailability normalised geomean EC10/NOECs for the species in the chronic Ni toxicity database (diamonds) in two European surface waters: Swedisch lake (left panel; pH 6.7; DOC 3.8 mg/L) and Ebro (right panel; pH 8.2; DOC 3.7 mg/L). Normalisations were conducted using the 'chronic Ni bioavailability and normalization tool' (Nys et al. 2016). Full lines represent the log-normal distribution function. Dashed lines denote the HC5, calculated using the method of Aldenberg & Jaworska (2000): 12.9 and 8.7 µg dissolved Ni/L in Swedisch lake & Ebro, respectively.

At the start of this study, bioavailability models for Pb were not yet available. Therefore, in the voluntary risk assessment report for Pb a Pb freshwater PNEC was derived without accounting

for the effects of water chemistry (LDAI 2008). One of the major recommendations of the European Union Scientific Committee on Human and Environmental Risks on the Pb risk assessment report was the use of bioavailability models for chronic toxicity for different species in the PNEC derivation (SCHER 2009). In the past years, chronic Pb bioavailability models have been developed for several species such as *C. dubia* (Chapter 2 of this study), fish (Van Sprang et al. 2016), and algae (De Schamphelaere et al. 2014). These bioavailability models have recently been used in a bioavailability-based European risk assessment for Pb (EC 2013b; Van Sprang et al. 2016).

1.6.3 Metal mixtures-regulation

Currently, possible mixture effects are not yet considered in European metal risk assessment frameworks, which may result in underestimation of the risks of metal exposure. However, it has been anticipated that future risk assessment procedures will require the consideration of mixture toxicity effects (SCHER 2009, CEU 2009). Over the last decade, several methods to evaluate mixture risks have been proposed which combine SSD-approaches with the CA and IA mixture reference models (Traas et al. 2002; De Zwart & Posthuma 2005; Van Regenmortel 2014). Recently, also a tiered approach to evaluate mixture risks has been presented by Backhaus & Faust (2012). It has been observed that the CA model is usually the most conservative model (e.g. Cedergreen et al. 2008; Kortenkamp et al. 2009). Additionally, predictions of the CA model fall mostly within a factor 2 of the observations (Belden et al. 2007; Cedergreen et al. 2008; 2014). Therefore, CA was proposed as a conservative first tier in tiered risk assessment approach (Backhaus & Faust 2012). In this tiered approach, the CA model is first used on the PNEC level and calculates a risk quotient for the mixture ($RQ_{PEC/PNEC}=\sum(PEC_i/PNEC_i)>$threshold?). In a subsequent tier, the CA model is applied on the toxicity data of the individual species, by calculating a risk quotient based on the summation of the 'species toxic units' (RQ_{STU}). For higher tiers, an IA based evaluation was proposed, if data suggests that the CA model would considerably overestimate mixture effects.

Noteworthy is the recent work of Van Regenmortel et al. (2014; 2015) who evaluated several mixture risk assessment methods (CA-SSD, IA-SSD, CA-DRC, and IA-DRC) on both simulated water chemistries characteristic for European surface waters and actual monitoring data. These

methods applied either CA or IA on the bioavailability normalised metal SSDs. In the CA-SSD, the toxic unit concept is applied on the HC5 of the metals in the mixture (Eq. 1.6). If the sum of TU, expressed relative to HC5 ($\sum TU_{HC5}$) is equal to 1 then 5% of the species in the community are considered to be potentially affected by the mixture.

$$\sum TU_{HC5} = \sum_{i=1}^{n} \frac{c_i}{HC5_i} \quad (1.6).$$

This approach can be considered equivalent to the application of the CA model to the PNEC proposed by Backhaus & Faust (2012). However, Van Regenmortel et al. (2014; 2015) preferred to use the metal concentrations and the HC5 instead of the PEC and PNEC, due to arbitrary assumptions involved in the calculation of the latter. The IA-SSD method, first proposed by De Zwart & Posthuma (2005) applies the IA model directly on the SSDs to predict the potentially affected fraction by the mixture (PAF_{Mix}). The CA-DRC applies the CA model directly to the species toxicity data in the SSD calculating a species specific TU_{EC10}, i.e. a toxic unit expressed relative to the 10% effective concentration (EC10; Eq. 1.3) The PAF_{Mix} is derived from the distribution of the species-specific TU_{EC10}. The CA-DRC approach is comparable by the RQ_{STU} evaluation suggested by Backhaus & Faust (2012). The IA-DRC approach calculates for every species the mixture effect based on Equation 1.5. Subsequently, the fraction of species that experience more than 10% effect is calculated, which is considered to be the PAF_{Mix}. The IA-DRC method, first proposed by Van Regenmortel et al. (2014), is the most complex method and requires data on the concentration response curves of each species.

Van Regenmortel et al. (2015) reported that the CA-SSD method was the most conservative approach if $\sum TU_{HC5}<1$. The IA-SSD is the most liberal method, i.e. least conservative and the CA-DRC and IA-DRC are intermediate methods. Based on these observations, a possible tiered metal mixture risk evaluation scheme was proposed (Van Regenmortel et al. 2015; Figure 1.7). In this tiered scheme, the CA-SSD was proposed as a conservative first tier and the IA-SSD as a more liberal subsequent tier. In a second tier, more advanced methods, such as the IA-DRC or CA-DRC or other approaches, were proposed.

Evaluation of monitoring data of environmental metal concentrations (Ni, Zn, Cu, and Cd) indicated that aquatic communities in some European regions (e.g. Flanders, Dommel river basin) are potentially at risk by metal exposure (Van Regenmortel et al. 2015). However, it was observed that in most cases this was due to the presence of one of the metals above its

individual HC5. The monitoring sites for which risks were predicted due to metal mixture exposure were in comparison rather limited.

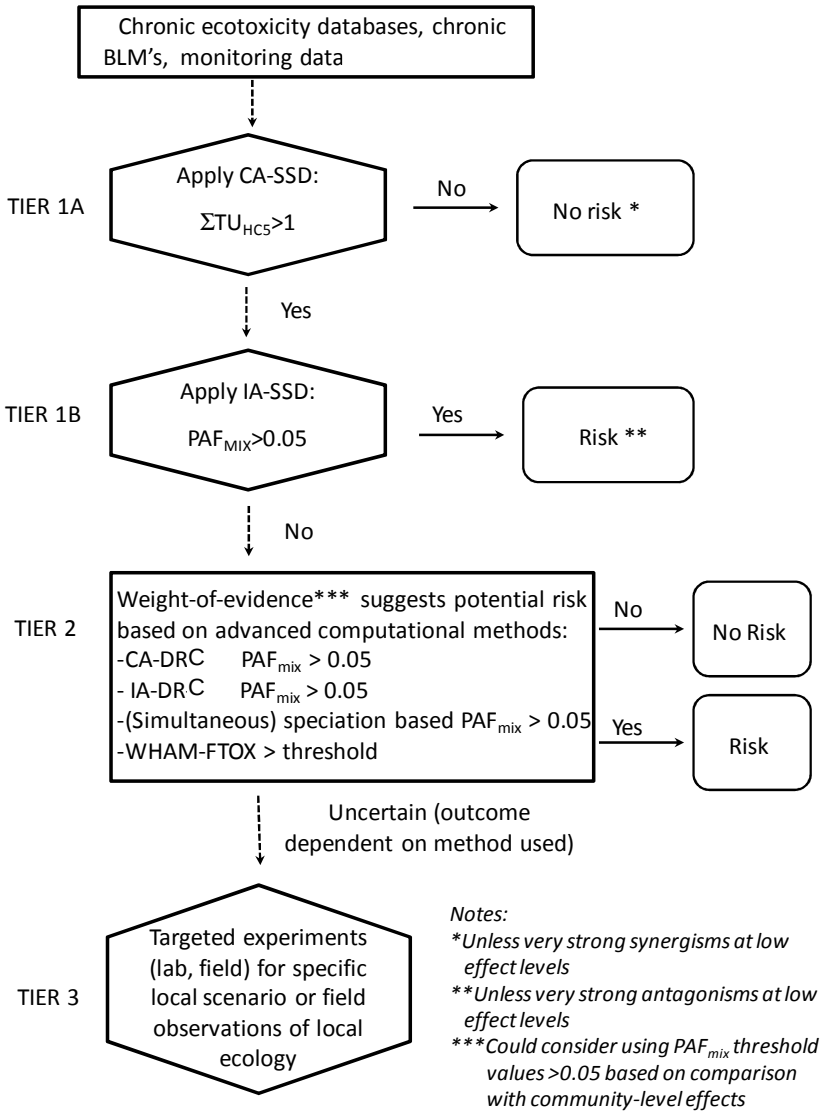

Figure 1.7. Possible tiered metal risk evaluation scheme proposed by Van Regenmortel et al. (2015). Figure adapted from Van Regenmortel et al. (2015).

1.7 Introduction to the test organisms- Daphnids

In the present study, metal bioavailability and metal mixture toxicity was investigated using the standard test species *C. dubia* and *D. magna* (Figure 1.8). These water fleas (Cladoceran) belong to the class of the Branchiopoda within the subphylum Crustacea.

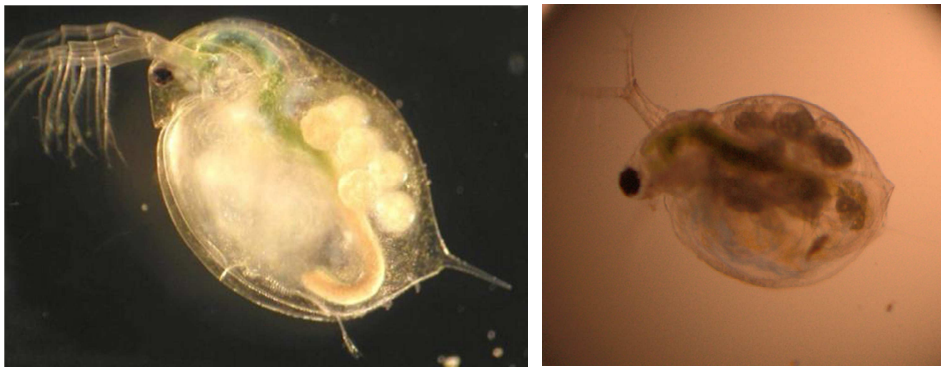

Figure 1.8. *Daphnia magna* (left; photo: Jennifer Hochmuth) and *Ceriodaphnia dubia* (right)

Cladocerans are small (0.5-10 mm) planktonic organisms (Koivisto 1995). A chitinous carapax, i.e. the exoskeleton, surrounds the thorax and abdomen. The head of Cladocerans usually carries several appendages: antennula (a sensory organ), antennae (swimming organs) and appendages functioning as mouth parts (Flössner 2000). The thorax carries five pairs of appendages which generate a water current into the ventral groove. This current is used to filter food particles, e.g. bacteria, protozoans, and algae, with the setulae on these limbs. Alternatively, the current enables gas exchange at the presumed respiratory structures, the epipodites (Pirow et al. 1999a; 1999b).

Cladocerans may reproduce either sexually or parthenogenetically (asexual). During the parthenogenetic phase, daphnid populations consist entirely of females. Eggs are released in the brood pouch under the female's caparax, where the embryogenesis occurs. After hatching from the eggs, the clonal young are released from the brood pouch during molting of the exoskeleton. Certain conditions, such as food limitation or a change in water temperature, induce sexual reproduction. In that situation, males will be released and females produce two haploid eggs, which are fertilized by the males. These ephippia further develop in the brood pouch and are released upon molting. The ephippia are resting eggs and may hatch when conditions are more favorable (Flössner 2000).

Cladocerans occur worldwide in all types of freshwater habitats, such as rivers, lakes, marches, springs, groundwater. Both *D. magna* and *C. dubia* occur in Europe, Asia, Africa, and North-America (Flössner 2000). *D. magna* live mainly in eutrophic ponds and rock pools in which planktivorous fish are absent (Koivisto 1995).

Due to their worldwide occurrence, short life cycle, parthenogenetic reproduction, ease to culture, small size, and central position in aquatic food webs, they are frequently used as model organisms in aquatic ecology (Lampert 2006) and ecotoxicology (Clesceri et al. 1998). Furthermore, they are rather sensitive to a broad range of aquatic contaminants (Clesceri et al. 1998), such as metals (Van Sprang et al. 2009; Schlekat et al. 2010; Esbaugh et al. 2012). Standard chronic ecotoxicity test methods have been developed by the Organisation for Economic Cooperation and Development (OECD 2002) and the United States Environmental Protection Agency (USEPA 2002a) for *D. magna* and *C. dubia,* respectively. The *D. magna* reproduction test lasts 21 days. However, in most chapters the *C. dubia* reproduction test was preferred due to the shorter exposure duration (mostly 7 days).

1.8 Conceptual framework of the study

Environmental Quality Standard for metals, such as Ni and Pb, in the WFD are now bioavailability based (EC 2013a). Additionally, bioavailability models have been considered in European risk assessment frameworks for several metals, such as Ni and Zn (DEPA 2008; Van Sprang et al. 2009). This all has increased the ecological relevance of metal EQS-derivations and European ecological risk assessment processes. However, metals mostly occur as mixtures in the environment. Current European environmental risk assessment frameworks do not yet account for the mixture effects which may arise from exposure to metal mixtures. Nevertheless, it has been anticipated that this will be necessary in the near future (SCHER 2009, CEU 2009). The absence of clear patterns emerging from metal mixture studies and the paucity of chronic metal mixture studies, currently hinders the development of metal mixture risk assessment frameworks (Van Genderen et al. 2015; Meyer et al. 2015a). Therefore, the present study aimed at investigating toxicity of Ni, Pb, and Zn mixtures during chronic exposure to daphnids. The reproducibility of mixture toxicity by either CA or IA is crucial for the development of future risk assessment procedures. It has been suggested that a priori knowledge of modes of action may

be used to select either IA or CA in those risk assessment procedures, although this remains to be tested for metal mixture toxicity. Nickel, Zn and Pb were selected because for daphnids different ions have been shown to compete with these metals at uptake sites (discussed above), which suggests the occurrence of dissimilar modes of action for these metals. Based on the latter, we hypothesised that the toxicity of their binary and ternary mixtures follows the IA model rather than the CA model. Additionally, we hypothesized that toxicity of Ni-Zn-Pb mixtures under varying water-chemistry can be predicted by a simple metal mixture bioavailability model that combines the bioavailability models of each of the individual metals with the IA model. Finally, we wanted to address the implications of our results for future metal mixture risk assessment processes.

The present study can be divided in four major parts (Figure 1.9). In a first part, the tools (i.e. BLMs or bioavailability models) for predicting individual metal toxicity were further refined and/or developed (Chapter 2-4). In a second part, chronic toxicity of Ni, Zn, and/or Pb mixtures to daphnid reproduction was investigated (Chapter 5-6). In a third part of this study, we developed and validated a chronic metal mixture bioavailability model for *C. dubia* (Chapter 7). Finally, we evaluated the implications of our chronic metal mixture toxicity results for metal mixture risk assessment processes (Chapter 8). Chapters 2-7 focused on metal (mixture) toxicity of the following three metals: Ni, Zn, and Pb. In Chapter 8, also other metal mixture combinations were considered. Each of the chapters contributes in its way to answer one of the above mentioned research hypotheses. (Figure 1.9)

At the start of the present study, chronic Pb bioavailability models were largely lacking, although a preliminary chronic *Ceriodaphnia* Pb bioavailability model existed (Esbaugh et al. 2012). However, there were still some unresolved issues about the effects of water chemistry parameters, such as Ca and pH, on chronic Pb toxicity to *C. dubia*. In **Chapter 2**, we investigated therefore the individual effects of water chemistry parameters such as Ca and pH on chronic Pb toxicity to *C. dubia*. Based on the results we developed a chronic *Ceriodaphnia* Pb BLM, which was extensively validated using chronic Pb toxicity data from several independent studies with *C. dubia*.

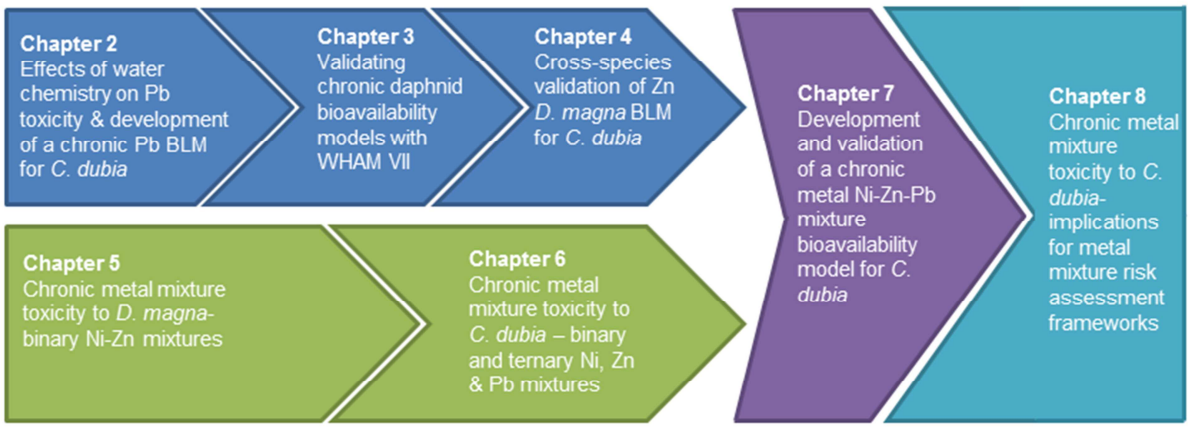

Figure 1.9. General overview of the framework of the study. In Chapter 2-4, the tools for predicting individual metal toxicity were further refined (blue figures). In Chapter 5 and 6, chronic toxicity of Ni, Zn, and/or Pb mixtures to daphnid reproduction was investigated. In Chapter 7, a chronic metal mixture bioavailability model for *C. dubia* was developed. In Chapter 8, the implications for metal mixture risk assessment processes were evaluated.

One of the major problems in integrating the existing chronic bioavailability models for individual metals into a chronic metal mixture bioavailability model, is that each model is currently based on different software to model metal speciation: e.g. WHAM V for Zn (Heijerick et al. 2005; De Schamphelaere et al. 2005), WHAM VI for Ni (De Schamphelaere et al. 2006; Deleebeeck et al. 2008) and Visual Minteq 3.0 for Pb (Chapter 2). Additionally, the assumptions for chemical speciation calculations differ between these metals. Recently, an updated version of the WHAM software including the Humic Ion-Binding Model VII (Tipping 2011) has been presented. WHAM VII can be considered as the state-of-the-art speciation software. Therefore, in **Chapter 3** we evaluated whether the chronic daphnid single metal bioavailability models can be updated to the WHAM VII speciation software.

At the start of the study, a chronic Zn *D. magna* BLM existed (Heijerick et al. 2005). However, the BLM was never validated for *C. dubia*. The integration of this BLM into a chronic Ni-Zn-Pb mixture bioavailability model for *C. dubia* (Chapter 7) requires that the applicability of this model to predict Zn toxicity to *C. dubia* is proven. In **Chapter 4**, we validated the chronic Zn *D. magna* BLM for *C. dubia* and developed a preliminary *C. dubia*-specific chronic Zn bioavailability model.

At the beginning of the study, the lack of chronic metal mixture toxicity data hindered the integration of metal mixture toxicity into risk assessment frameworks (Van Genderen et al. 2015).

In a first step to assess chronic metal mixture toxicity to daphnids, we investigated in **Chapter 5** the interactive effects of the binary Ni-Zn mixture on *D. magna* reproduction.

In **Chapter 6**, we further investigated the effects of metal mixture toxicity to daphnids. However, the experiments were conducted using *C. dubia* as test organisms. The *C. dubia* exposure duration in standard chronic toxicity tests is considerably shorter than for *D. magna* (7 days vs. 21 days). The *C. dubia* reproduction test allows a more efficient screening of metal mixture toxicity. In **Chapter 6**, we investigated interactive mixture effects of Ni, Zn, and Pb in binary and ternary mixture combinations.

In **Chapter 7**, we investigated whether chronic metal mixture toxicity to *C. dubia* can be predicted using a chronic metal mixture bioavailability model. A mixture bioavailability model was developed by combining the existing chronic bioavailability models for individual metal toxicity (Chapter 2; Chapter 4 and De Schamphelaere et al. 2006) with the IA model (based on results from chapter 6). The model was validated using Ni-Pb-Zn mixture toxicity data in (modified) natural waters.

In **Chapter 8**, we combined all metal mixture toxicity data for *C. dubia* from the present study and one additional study (Nys et al. under review) in a meta-analysis. We evaluated the implications of our mixture toxicity data for metal risk assessment frameworks. Finally, a possible tiered metal mixture risk evaluation approach is proposed and evaluated using environmental monitoring data.

2

Development and validation of a biotic ligand model for predicting chronic toxicity of lead (Pb) to *Ceriodaphnia dubia*

Redrafted from:

Nys C, Janssen CR, Mager EM, Esbaugh AJ, Brix KV, Grosell M, Stubblefield WA, Holtze K, De Schamphelaere KAC. 2014. Development and validation of a biotic ligand model for predicting chronic toxicity of lead to *Ceriodaphnia dubia*. *Environmental Toxicology and Chemistry* 33:394–403.

2. Development and validation of a biotic ligand model for predicting chronic toxicity of lead (Pb) to *Ceriodaphnia dubia*

2.1 Introduction

Lead is a non-essential metal which can be harmful to aquatic organisms even at low concentrations (Grosell et al. 2006b; Mager et al. 2011a; Esbaugh et al. 2012). As with many other metals, the bioavailability and consequently also the toxicity of Pb to freshwater organisms is dependent on the physicochemical characteristics of the water (Mager et al. 2011a; 2011b; Grosell et al. 2006a). The Biotic Ligand Model (BLM) provides a framework to account for the influence of water chemistry on metal toxicity. The BLM concept starts from the principle that the toxicity of a metal is dependent on the concentration of the metal bound to the biotic ligand, i.e., a receptor at the cell surface, and the activity of certain cations (e.g., H^+, Ca^{2+}), which compete with the metal ion for binding sites at the biotic ligand (Di Toro et al. 2001). When the concentration of metal bound to the biotic ligand transcends a certain critical concentration, a toxic effect will occur.

In the last decade, chronic BLMs were developed for several metals including Cu (e.g., De Schamphelaere & Janssen 2004a), Zn (e.g., Heijerick et al. 2005) and Ni (e.g., Deleebeeck et al. 2008). Furthermore, BLMs are becoming more integrated into risk assessment procedures in regions such as Europe (e.g., Van Sprang et al. 2009) and the United States (USEPA 2007) but efforts to develop chronic Pb BLMs were until now limited. Recently, a preliminary chronic Pb BLM for *Ceriodaphnia dubia* was developed (Esbaugh et al. 2012). This preliminary BLM was based on the data of Mager et al. (2011a) and a study conducted by Parametrix (2010). However, these studies could not unambiguously establish the individual effects of Ca and pH as the effects differed between these studies. First, Mager et al. (2011a) did not find a protective effect of $CaSO_4$ additions on chronic Pb toxicity to *C. dubia*. They also found that pH affected Pb toxicity, but they noted that their results may have been confounded by the addition of the pH buffer MOPS (Esbaugh et al. 2013). In contrast, Parametrix (2010) reported that increased ambient Ca reduced chronic Pb toxicity to *C. dubia*. However, this increase of Ca was accompanied by increases of pH and alkalinity. Furthermore, speciation calculations predicted colloidal precipitation of (hydro)cerussite and leadhydroxide in three of the four Ca

treatments. Hence, it could not be unambiguously concluded if the effect observed was due to increased Ca. In addition, the same study reported highest toxicity at intermediate pH (between pH 7 and 8) and lower toxicity at pH 6 and pH 8.5. Considering all results together, there is no unifying explanation for the differences observed between these two studies.

Given these uncertainties, it is unclear whether the preliminary Pb BLM accurately depicts the bioavailability processes concerning Pb. This hinders the development of a definitive chronic BLM for *Ceriodaphnia* which has been used as a model species for all invertebrates for the normalization of toxicity data in risk assessment processes (Schlekat et al. 2010). The purpose of the present study was to more clearly define the individual effects of Ca and pH on chronic Pb toxicity to *C. dubia.* Therefore, we performed chronic reproduction tests with *C. dubia* wherein Ca and pH were modified independently of one another. Furthermore, all tests within a test series were run simultaneously to avoid temporal shifts in toxicity. Based on the obtained data a final chronic Pb BLM for *C. dubia* was developed. Finally, the developed BLM was validated with several available data sets originating from chronic Pb toxicity studies with *C. dubia* in both synthetic and field collected natural waters (Parametrix 2010; Mager et al. 2011a; Esbaugh et al. 2012, AquaTox 2012).

2.2 Material & methods

2.2.1 Collection and preparation of test media

All toxicity tests were conducted in modified natural water. The natural water was collected from L'Ourthe Orientale in Brisy, Belgium. This unpolluted water has previously been used successfully for ecotoxicity testing in our laboratory (e.g., Deleebeeck et al. 2008) and has a low hardness and Dissolved Organic Carbon (DOC) concentration. The natural water was filtered on site through a 0.2 µm filter and stored at 4°C in total darkness in 10L acid washed polyethylene barrels until use. The pH of the Brisy water was 6.7 at the time of sampling. The Ca, Mg, Na, Cl and SO_4^{2-} concentrations were 0.25, 0.16, 0.28, 0.41 and 0.10 mM, respectively. Filtered Pb concentrations were below the detection limit (DL_{Pb}=0.4 µg Pb/L). The DOC concentration was 3.2 mg/L.

The individual effects of Ca and pH on chronic Pb toxicity were investigated in two univariate test series, each consisting of four treatments (i.e., four exposure media). In the Ca series, Ca concentration was varied by adding $CaCl_2$ to the Brisy water. Four Ca concentrations were investigated; 0.25 mM Ca (unmodified Brisy water), 1 mM Ca (addition of 0.75 mM $CaCl_2$), 1.75 mM Ca (addition of 1.5 mM $CaCl_2$) and 2.5 mM Ca (addition of 2.25 mM $CaCl_2$). All media in the Ca series were adjusted to pH 7 by adding dilute NaOH. In the pH series 4 pH levels were investigated: 6.4, 7.0, 7.6 and 8.2. To all media in the pH series 0.5 mM $CaCl_2$ was added. Initially, pH was adjusted by adding 0.4 and 2.6 mM $NaHCO_3$ to pH level 7.6 and 8.2, respectively. Na levels were set equal between pH treatments by adding Na_2SO_4. All media were aerated for three days to allow equilibration with the ambient atmosphere. Subsequently, pH was adjusted to the required pH level by adding dilute HCl or NaOH before dividing the medium in aliquots of 1.6L. The ultimate physicochemical composition of the different test media is shown in Table 2.1.

Table 2.1.- Main physicochemical characteristics of the test media used for biotic ligand model development for chronic Pb toxicity to *Ceriodaphnia dubia* . Values are arithmetic means of all measurements ±standard deviation.

Test medium	pH	DOC (mg/L)	Ca (mM)	Mg (mM)[a]	Na (mM)[a]	SO_4 (mM)[a]	Cl (mM)[a]	DIC (mM)	Hardness[b] (mg $CaCO_3$/L)
Ca 0.25 mM	7.04±0.06	4.0±1.1	0.24±0.01	0.16	0.32	0.10	0.41	0.20±0.04	40.0
Ca 1.0 mM	7.01±0.05	3.9±1.0	0.89±0.18	0.16	0.32	0.10	1.91	0.17±0.04	105
Ca 1.75 mM	7.04±0.06	3.8±1.0	1.26±0.09	0.16	0.31	0.10	3.41	0.18±0.03	142
Ca 2.5 mM	7.07±0.09	3.9±0.8	1.80±0.06	0.16	0.30	0.10	4.91	0.19±0.04	196
pH 6.4	6.35±0.26	3.3±0.5	0.75±0.01	0.16	2.89	1.40	2.03	0.07±0.04	91.1
pH 7.0	6.94±0.10	3.3±0.5	0.77±0.02	0.16	2.90	1.40	1.93	0.17±0.02	93.1
pH 7.6	7.56±0.10	3.3±0.6	0.74±0.01	0.16	2.96	1.20	1.91	0.53±0.03	90.1
pH 8.2	8.14±0.04	3.2±0.3	0.74±0.02	0.16	2.93	0.10	1.95	2.33±0.08	90.1

[a] No measurements were conducted for Mg, Na, SO_4 and Cl. The baseline concentrations measured in the Brisy water at the time of sampling are reported. For Na SO_4 and Cl additions of $NaHCO_3$, $NaSO_4$, $CaCl_2$, NaOH and HCl were taken in to account (see materials and methods).

[b] Water hardness was calculated from measured Ca and Mg (at time of sampling) concentrations

DOC=dissolved organic carbon; DIC= dissolved inorganic carbon

Concentration series in each Ca and pH treatment contained a control and 6 Pb concentrations, which were prepared by adding $PbCl_2$. For the Ca test series the nominal Pb concentrations were 50, 100, 150, 220, 320 and 400 µg Pb/L. For the pH series the nominal Pb concentrations were 80, 110, 140, 170, 220 and 320 µg Pb/L for pH levels 6.4, 7.0 and 7.6 and 100, 160, 220, 280, 340 and 400 µg Pb/L for pH level 8.2. All chemicals were purchased from VWR International.

2.2.2 Ecotoxicity testing

The chronic Pb toxicity tests were conducted following the USEPA protocol (USEPA 2002a). *C. dubia* juveniles originated from an in-house isoclonal lab culture which has been maintained for more than 20 years at 25°C in carbon filtered Ghent city tap water to which selenium (1 µg Se/L) and vitamins (75 µg/L thiamine, 1 µg/L cyanocobalamine, 0.75 µg/L biotine) are added. Daphnids were acclimated to test media in aquaria containing 1.5L of the control test media (no Pb) for two generations prior to test initiation (2 weeks). Media were refreshed twice a week. During the acclimation and the testing period, daphnids were fed with *Pseudokirchneriella subcapitata* algae (2 10^5 cells/mL) and YUT-mixture (Yeast-Urtica-Trout Chow mixture of 12 mg solids/L). The four ecotoxicity tests per test series were all run simultaneously to exclude any possible interference with later data interpretation due to temporal sensitivity variation. Tests were initiated with juveniles of the second generation from "mothers" that produced at least 8 juveniles in a single brood (USEPA 2002a). Juveniles (<24h old, 1 per replicate) were randomly distributed among 10 replicates for each control and Pb concentration. Tests were conducted in polyethylene cups containing 20 mL of test medium at 25°C under a light cycle of 16 h light and 8 h dark. Test media were completely renewed daily. Before renewal, fresh test media were adjusted to the required pH by adding dilute HCl or NaOH. Mortality and number of juveniles were scored daily. The toxicity tests were ended when 60% of the control animals had produced three broods (7 to 8 days).

2.2.3 Analytical chemistry

During the test period, samples of fresh (sample of new medium just before transfer of daphnids to the cup) and old (sample taken of medium just after transfer of daphnids to a new cup) test media were collected regularly for analysis of total Pb and filtered (0.45 µM, Acrodisc, PALL Life Sciences) Pb, Ca, OC (organic carbon) and IC (inorganic carbon). Total and filtered samples of fresh media were taken on days 0, 3 and 6. Filtered samples of old media were taken on days 1, 4 and 7. Samples for Pb and Ca measurements were acidified to 0.14 mol/L HNO_3 (Normatom quality, VWR Prolabo). Pb concentrations were measured using graphite furnace atomic absorption spectrophotometry (GFAAS Furnace Autosampler, Thermo Fisher Scientific Inc). Ca concentrations were measured using flame atomic absorption spectrophotometry (SpectrAA100, Varian). DOC (Dissolved Organic Carbon) and DIC (Disolved Inorganic Carbon)

were measured with a Total Organic Carbon analyzer (TOC-5000, Shimadzu). The pH of fresh and old media were measured daily with a pH glass electrode (P407, Consort).

2.2.4 Concentration response analysis

Effect concentrations (NOEC, LOEC, EC10, EC20 and EC50) were calculated based on average measured filtered Pb concentrations in fresh and old test media. Total reproduction (number of juveniles per female) relative to the mean control reproduction (%) was used as the endpoint. NOECs and LOECs were determined with the Mann-Whitney U test with Bonferroni-Holm correction in the software package SPSS 20 (SPS Inc). $EC50_{Pbfilt}$, $EC20_{Pbfilt}$, $EC10_{Pbfilt}$ and corresponding confidence intervals were determined with the drc-package in R 2.14.1 (R Development Core Team, Vienna, Austria) with a log-logistic concentration response model with two parameters (Eq. 2.1)

$$y = \frac{100}{1+exp^{(b(\log(x)-\log(ECx)))}} \quad (2.1)$$

Where y= predicted reproduction (number of offspring per female) relative to the average of the controls (%), b= a slope parameter, x= the filtered Pb concentration (µg/L), and ECx_{Pb}= the effect (10, 20 or 50%) concentration (µg filtered Pb/L).

2.2.5 Chemical speciation calculations

Pb^{2+}, Ca^{2+} and H^{+} activities were calculated using Visual Minteq 3.0 (KTH, Stockholm, Sweden). Speciation was calculated at the $EC50_{Pbfilt}$ and $EC20_{Pbfilt}$. Complexation of Pb with dissolved organic matter (DOM), i.e., with humic acid (HA) and fulvic acid (FA), was modeled according to the NICA-Donnan formulation (as embedded in Visual Minteq 3.0). All default parameter values as described in Milne et al. (2003) were used. In all cases we assumed that DOM contains 50% carbon on a weight basis. For DOM from natural sources (e.g., tap water, surface water) we assumed that 65% of the DOM is reactive and behaves as isolated FA. Previous research has shown that assumptions between 60% and 70% active FA typically work best for predicting metal toxicity in natural waters (Tipping 2002). Accordingly, the measured DOC content (mg C/L) for natural sources was multiplied by a factor of 1.3 to obtain the amount of FA (mg FA/L) to be used as the modeling input. For four tap waters to which (isolated) Aldrich HA (AHA) was added (i.e., media in Mager et al. 2011a) we assumed that this HA behaved as 100% reactive

HA. However, it was taken into account that there was a natural background DOM concentration present in the tap water. Thus, in these cases, the HA concentration (mg HA/L) was estimated as 2 fold (measured DOC – measured background DOC) and the FA was 1.3 fold the background DOC concentration.

It needs to be mentioned that the publically available Visual Minteq 3.0 contains an error in the speciation calculation code which results in errors in the calculation of Pb speciation when two or more different sources of organic matter are present in a medium (in this case FA and HA) in the input file. Speciation calculations in the present study were therefore made in an adapted version obtained after personal communication with Jon Petter Gustafsson (KTH, Stockholm, Sweden), which is not yet available online.

The model development and validation was always based on (average) measured filtered concentrations of Pb, operationally defined as the Pb passing through a 0.45 µm filter. Yet, it is possible that not all filtered Pb is 'truly dissolved' because colloidal precipitates of Pb minerals may be present in the test solutions. However, in the absence of any measured data on the presence of such colloidal precipitates, we assumed for modeling purposes (i.e., model development and validation) that all filtered Pb was truly dissolved and could interact with dissolved ligands (e.g. DOC) in the medium. Thus, the average of measured filtered Pb concentrations of new and old media were used as both the input and the output of the BLM (see further) for both development and validation. We explored also the potential importance of colloidal Pb precipitation in reducing Pb bioavailability within the filtered Pb fraction. This was achieved by performing additional speciation calculations where in Visual Minteq 3.0 the formation of the Pb-minerals cerrusite, hydrocerrusite (two Pb-carbonate minerals), and $Pb(OH)_2(s)$ were allowed. In all test media investigated here, no other Pb-minerals were ever predicted to form. We anticipated that these additional calculations could provide insight on the role colloidal Pb mineral formation may play in the observed variation of measured filtered EC50 among test media.

2.2.6 BLM development

BLM development was based on $EC50_{Pb2+}$, since EC50 give more precise estimates (smaller confidence intervals) than EC20 and EC10. The effects of Ca and pH on chronic Pb toxicity to *C. dubia* were modeled as a single-site BLM-type competition effect according to the method described by De Schamphelaere & Janssen (2002). BLM parameters (K_{HBL} and K_{CaBL}) were determined based on regression equations of the linear relationship between 7d-$EC50_{Pb2+}$ and H^+ and Ca^{2+} activity, respectively. The linear relationships were evaluated by an F-test using a significance level of 5% with R 2.14.1 (R Development Core Team, Vienna, Austria). Estimated intercept and slope parameters with corresponding standard errors are reported. The 95% confidence intervals on the BLM parameters were calculated based on the standard error of the ratio between the slope and the intercept of the regression equation. The lower and upper limits of the 95% confidence intervals were calculated as the K_{HBL} +/-1.96xstandard error, respectively.

The BLM equation including a Ca^{2+} and a H^+ competition effect can be written as follows

$$EC50_{Pb^{2+},i,predicted} = EC50^*_{Pb^{2+}} \times (1 + K_{HBL} \times \{H^+\}_i + K_{CaBL} \times \{Ca^{2+}\}_i) \quad (2.2)$$

In this model $EC50_{Pb2+,i,predicted}$ is the predicted Pb^{2+} activity for the 50% effect concentration in test solution *i*. K_{HBL} and K_{CaBL} are the stability constants for binding of H^+ and Ca^{2+} to the biotic ligand for Pb toxicity and $\{H^+\}_i$ and $\{Ca^{2+}\}_i$ are the chemical activities of H^+ and Ca^{2+} in test solution *i*, respectively. $EC50^*_{Pb2+}$ is the intrinsic sensitivity of *C. dubia*, which can be regarded as the $EC50_{Pb2+}$ of *C. dubia* in a solution where all H^+ and Ca^{2+} competition effects are absent (De Schamphelaere & Janssen 2002). With the above model $EC50_{Pb2+}$ of any test solution can be predicted, if K_{HBL} and K_{CaBL} are known. The intrinsic sensitivity can be estimated from observations of EC50 in a range of waters as follows:

$$EC50^*_{Pb^{2+}} = \prod_i^n \left(\frac{EC50_{Pb^{2+},i,observed}}{1 + K_{HBL} \times \{H^+\}_i + K_{CaBL} \times \{Ca^{2+}\}_i} \right)^{1/n} \quad (2.3)$$

Where $EC50_{Pb2+,i,\ observed}$ is the Pb^{2+} activity in the test solution *i* and *n* is the number of test solutions considered.

The predicted 7d-$EC50_{Pb2+}$ were translated to 7d-$EC50_{Pbfilt,pred}$ with Visual Minteq 3.0 and compared with the observed 7d-$EC50_{Pbfilt,obs}$. The developed BLM was also auto-validated with the

7d-$EC10_{Pbfilt}$ and 7d-$EC20_{Pbfilt}$, following the procedure described above, but calibrating the intrinsic sensitivity specifically for $EC10_{Pbfilt}$ and $EC20_{Pbfilt}$.

2.2.7 Independent BLM validation

The developed chronic Pb BLM was validated with Pb toxicity data from the following four independent datasets obtained in earlier studies. Mager et al. (2011a) investigated the effect of modifications of major cation concentrations (Ca, Mg, Na, K), pH (using MOPS buffered media), alkalinity ($NaHCO_3$ additions) and DOC (added as natural organic matter, NOM or as Aldrich HA). A total of 21 test media were investigated. The water chemistry and corresponding 7d-$EC50_{Pbfilt}$ and 7d-$EC20_{Pbfilt}$ were taken from their Table 1. Esbaugh et al. (2012) reported on the chronic toxicity of Pb in 5 spiked natural surface waters. The water chemistry and corresponding 7d-$EC50_{Pbfilt}$ and 7d-$EC20_{Pbfilt}$ was taken from their Table 1 and Table 2, respectively. Two additional datasets were used. Parametrix (2010) investigated the effects of pH and Ca. Chemical composition of the test media were taken from their Tables 3-1 and 3-2. The 7d-$EC50_{Pbfilt}$ and 7d-$EC20_{Pbfilt}$ were taken from their Tables 3-9 and 3-10. AquaTox (2012) retested the French Lake natural water, which was identified as an outlier of a previous preliminary BLM study (Esbaugh et al. 2012). Additionally, a synthetic lab water and a reconstituted 'French Lake' water were used to investigate the effect of Pb to *C. dubia*. Chemical characteristics were taken from their Table 3. The 7d-$EC50P_{bfilt}$ and the 7d-$EC20_{Pbfilt}$ were taken from their Table 19. An overview of the chemical composition of the different test media used for model validation is given in *Appendix A* (Table A.1).

The validation of the developed BLM was performed in two different ways. First, a validation was performed by calculating a single mean intrinsic sensitivity ($EC50^*_{Pb2+}$ and $EC20^*_{Pb2+}$) for all validation datasets and the data reported in this study with Equation 3, hereafter called the "overall intrinsic sensitivity". A second validation was made by calculating a mean intrinsic sensitivity, ($EC50^*_{Pb2+}$ and $EC20^*_{Pb2+}$), for each *C. dubia* clone separately with Equation 3, hereafter called the "clone-specific intrinsic sensitivity". Mager et al. (2011a) and Esbaugh et al. (2012) tested Pb toxicity on the same *C. dubia* clone, hereafter called the "UMiami clone". Therefore a clone-specific intrinsic sensitivity was used for these two datasets together.

In the calculation of the overall intrinsic sensitivity, the 0.25 mM Ca treatment of the present study was excluded. The following data points were excluded in the calculation of the intrinsic sensitivity in both validation analysis: for the dataset of Parametrix (2010) the four data points for which speciation calculations predicted colloidal precipitation (see further) were excluded from the calculation of the intrinsic sensitivities. For Mager et al. (2011a), the synthetic waters with added humic acid (HA) and those buffered with MOPS were excluded from the calculation of the intrinsic sensitivities, because such additions represent conditions that are less relevant for natural waters. Furthermore, the French Lake water was also excluded from the calculations, because Esbaugh et al. (2012) showed that Pb toxicity in the French Lake water was underestimated by 12-fold using a preliminary *C. dubia* BLM. For the AquaTox (2012) dataset, calibration of the intrinsic sensitivity was done based on all three tested waters. The French Lake water was tested by both Esbaugh et al. (2012) and AquaTox (2012). Hence, for clarity, the French Lake water tested by Esbaugh et al. (2012) and AquaTox (2013) will herein be called French Lake 1 and French Lake 2, respectively.

The developed BLM was not validated with $EC10_{Pbfilt}$, as these were not available for all datasets. Furthermore, EC10 are usually less precise than EC20 or EC50 (larger confidence intervals).

2.3 Results

2.3.1 Effect of Ca and pH on Pb toxicity

No colloidal Pb precipitation was predicted in the Ca or pH test series. Control survival was 100% in all Ca treatments. Control reproduction in the Ca test series was on average 14.0 (±4.7) juveniles per female (±st. dev) (Table 2.2). In the controls of the three highest Ca concentrations (1.00-1.75 mM), we observed ≤10% males, while 30% males were observed in the control of the 0.25 mM Ca treatment. The concentration response data of the Ca test series are shown in Figure 2.1.A. No reliable log-logistic concentration response curve could be fitted for the 1.0 and 2.5 mM Ca treatments. The $EC50_{Pbfilt}$ for these treatments were therefore estimated by linear regression between the observed effect (%) at the two concentrations encompassing the 50% effect levels and the log filtered concentration (µg Pb/L). The $EC50_{Pbfilt}$ were similar at the three highest Ca concentrations (Table 2.2), i.e., 104-130 µg/L, but were 1.3 to 1.6-fold lower at the 0.25 mM Ca concentration. Figure 2.2.A shows the positive linear relationship

between 7d-$EC50_{Pb2+}$ and Ca^{2+} activity (p=0.22, r=0.78). The estimated slope of this relationship was 4.79×10^{-6} ($\pm 2.70 \times 10^{-6}$). The intercept of the relation was 4.23×10^{-9} mol/L($\pm 2.27 \times 10^{-9}$).

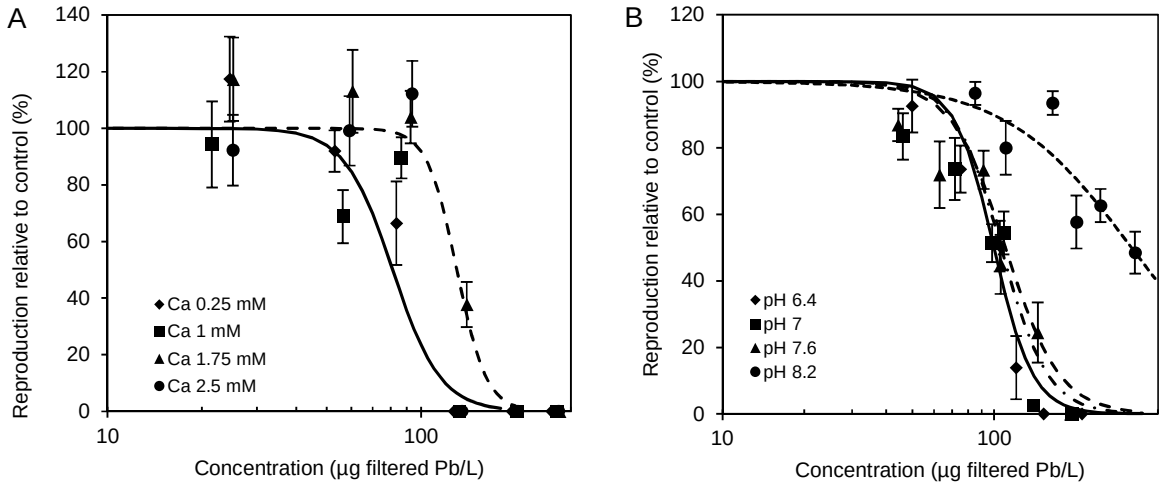

Figure 2.1. Concentration response curves of Pb toxicity to *Ceriodaphnia dubia* for the Ca test series (A) and the pH test series (B): Data points are the mean reproduction relative to the control reproduction. Plotted error bars are standard errors. Fitted curves are log-logistic concentration response curves (Eq. 2.1): Ca 0.25 mM and pH 6.4 (Full line), pH 7 (Dashed-dotted line), Ca 1.75 mM and pH 7.6 (Dashed line), pH 8.2 (Dotted line). No reliable log-logistic concentration response curve could be fitted for the 1.0 and 2.5 mM Ca treatments.

Control females in the pH test series produced on average 23.2±5.5 juveniles (mean±st. dev, Table 2.2). No mortality or males were observed in the controls of all pH treatments. The concentration response data of the pH test series are shown in Figure 2.1.B. Lead toxicity expressed as the average filtered concentration in the new and old media was similar between pH 6.4 and pH 7.6. Lead toxicity at pH 8.2 was clearly lower. The 7d-$EC50_{Pbfilt}$ varied at most 1.1-fold between pH 6.4 and 7.6 (i.e., 100-110 µg/L), while a pH change from pH 7.6 to 8.2 resulted in a 2.9-fold increase of the 7d-$EC50_{Pbfilt}$ (Table 2.2). Figure 2.2.B shows the positive linear relationship between 7d-$EC50_{Pb2+}$ and H^+ activity (p=0.003, r=0.99). The estimated slope of this relationship was 6.25×10^{-2} ($\pm 3.25 \times 10^{-3}$). The intercept of the relation was 1.63×10^{-9} mol/L($\pm 7.58 \times 10^{-10}$).

NOEC and LOEC values are provided in Table 2.2. The NOEC and LOEC at 0.25 mM Ca are less reliable as already 33% effect was observed at the NOEC. The NOECs and LOECs of the other

treatments show the same trends as the regression based effect concentrations, with higher NOEC and LOEC at high pH and no effect of Ca.

Table 2.2. General biological characteristics of the toxicity tests with *Ceriodaphnia dubia*: Mean control reproduction (number of juveniles per mother animal ± standard deviation, percentage of males in control (%), 50%, 20% and 10% effect concentrations, No Observed Effect Concentration and Lowest Observed Effect Concentration for the reproduction endpoint in terms of µg dissolved Pb/L). For EC50s, EC20s and EC10s, 95% confidential intervals are reported between brackets. For NOECs and LOECs, reduction in reproduction relative to the control (%) are reported between brackets.

Test medium	Control reproduction	Percentage of males in control	EC50 (µg Pb_{filt}/L)	EC20 (µg Pb_{filt}/L)	EC10 (µg Pb_{filt}/L)	NOEC (µg Pb_{filt}/L)	LOEC (µg Pb_{filt}/L)
Ca 0.25 mM	14.1±4.6	30%	81.2 (66.6-95.8)	64 (42-86)	55 (30-81)	83 (-33%)	127 (-100%)
Ca 1.0 mM	14.4±4.2	10%	104[a] (86.5-132)	a	a	86 (-10%)	132 (-100%)
Ca 1.75 mM	13.9±4.4	0%	130 (110-150)	112 (80-144)	102 (63-142)	93 (-0%)	140 (-62%)
Ca 2.5 mM	13.6±6.3	10%	115[a] (93.7-135)	a	a	94 (-0%)	135 (-100%)
pH 6.4	21.3±7.5	0%	99.8 (87.6-112)	79 (60-98)	70 (48-92)	50 (-7%)	75 (-26%)
pH 7	22.4±5.0	0%	106 (94.7-118)	81 (61-101)	69 (45-93)	46 (-17%)	72 (-26%)
pH 7.6	23.9±5.4	0%	110 (95.8-124)	80 (58-102)	67 (41-92)	44 (-13%)	63 (-28%)
pH 8.2	25.0±5.8	0%	320 (242-398)	153 (87-129)	99 (34-165)	164 (-7%)	201 (-42%)

[a] Due to the steepness of the concentration response no reliable EC50, EC10 and EC20 could be calculated for the 1 and 2.5 mM Ca treatment with the log-logistic dose response. EC50s were derived from the regression between the observed effect (%) at the two concentrations encompassing the 50% effect level and the log filtered concentration (µg Pb/L). The reported confidence limits for these tests are the two concentrations which encompass the 50% effect levels.

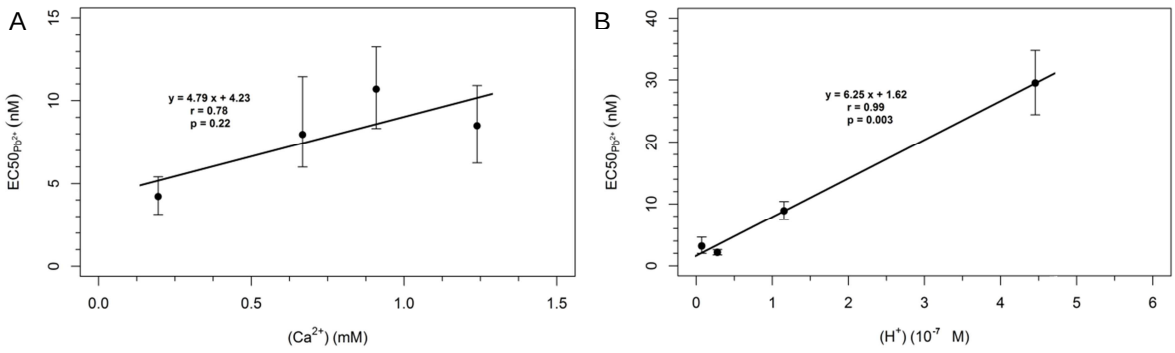

Figure 2.2. $EC50_{Pb2+}$ (expressed as Pb^{2+} activity, µM) as a function of Ca^{2+} activity (mM, A) and H^+ activity (10^{-7} M, B) for *Ceriodaphnia dubia*. The regression line, equation and p value are given. Plotted error bars are 95% confidence intervals on the $EC50_{Pb2+}$.

2.3.2 Model development

Based on the results from the univariate pH and Ca test series, a chronic Pb BLM was developed. A stability constant for Ca (log K_{CaBL}) was not incorporated into the BLM, because of reasons discussed further on *(see Discussion section)*. In absence of other competing ions K_{HBL} can be estimated by the ratio of the slope to the intercept of $EC50_{Pb2+}$ as a function of H^+ activity (De Schamphelaere & Janssen 2002). Log K_{HBL} was calculated to be 7.6 (95% CI: 6.5-7.9). Without the non-significant Ca competition term the BLM can be written as follows

$$EC50_{Pb^{2+},i,predicted} = EC50^{*}_{Pb^{2+}} \times (1 + K_{HBL} \times \{H^+\}_i) \quad (2.4)$$

An auto validation was done to investigate how well the model predicts Pb toxicity for the dataset used for BLM development. Using Equation 2.3 mean clone-specific intrinsic sensitivities ($EC50^{*}_{Pb2+}$, $EC20^{*}_{Pb2+}$ and $EC10^{*}_{Pb2+}$) of 1.73, 1.01 and 0.75 mM, respectively, were calculated based on all data points (except that for 0.25 mM Ca, for reasons discussed further on). For the $EC50_{Pbfilt}$, all predicted values were within 1.5-fold of those observed, except for the 0.25 mM Ca treatment (Figure 2.3). For the $EC20_{Pbfilt}$, all predicted values were within 1.5-fold of those observed. Finally, for the $EC10_{Pbfilt}$, all predicted values were predicted within 2-fold of the observed values (Appendix A, Table A.6 & A.7).

2.3.3 Independent model validation

The overall intrinsic sensitivities were estimated at 1.33 and 0.54 nM for $EC50^{*}_{Pb2+}$ and $EC20^{*}_{Pb2+}$, respectively. Using these overall intrinsic sensitivities, 7d-$EC50_{Pbfilt}$ in synthetic waters were predicted with an average error of 2.11 (Figure 2.3.A), while 7d-$EC20_{Pbfilt}$ in synthetic waters were predicted with an average error of 2.14. The $EC50^{*}_{Pb2+}$ clone-specific intrinsic sensitivities were estimated at 3.06, 1.06 and 0.94 nM for the Parametrix, Miami and AquaTox clone, respectively, while the $EC20^{*}_{Pb2+}$ were 1.40, 0.44 and 0.17 nM, respectively. Using these clone-specific intrinsic sensitivities, both 7d-$EC50_{Pbfilt}$ and 7d-$EC20_{Pbfilt}$ in the synthetic waters were predicted with an average error of 1.89 (Figure 2.3.B). All overall and clone-dependent intrinsic sensitivities and average prediction errors of the 7d-$EC50_{Pbfilt}$ and 7d-$EC20_{Pbfilt}$ for the different types of waters are summarized in Appendix A (Table A.2-4). The predicted 7d-$EC50_{Pbfilt}$ and 7d-$EC20_{Pbfilt}$ for all waters are provided in Appendix A (Table A.6).

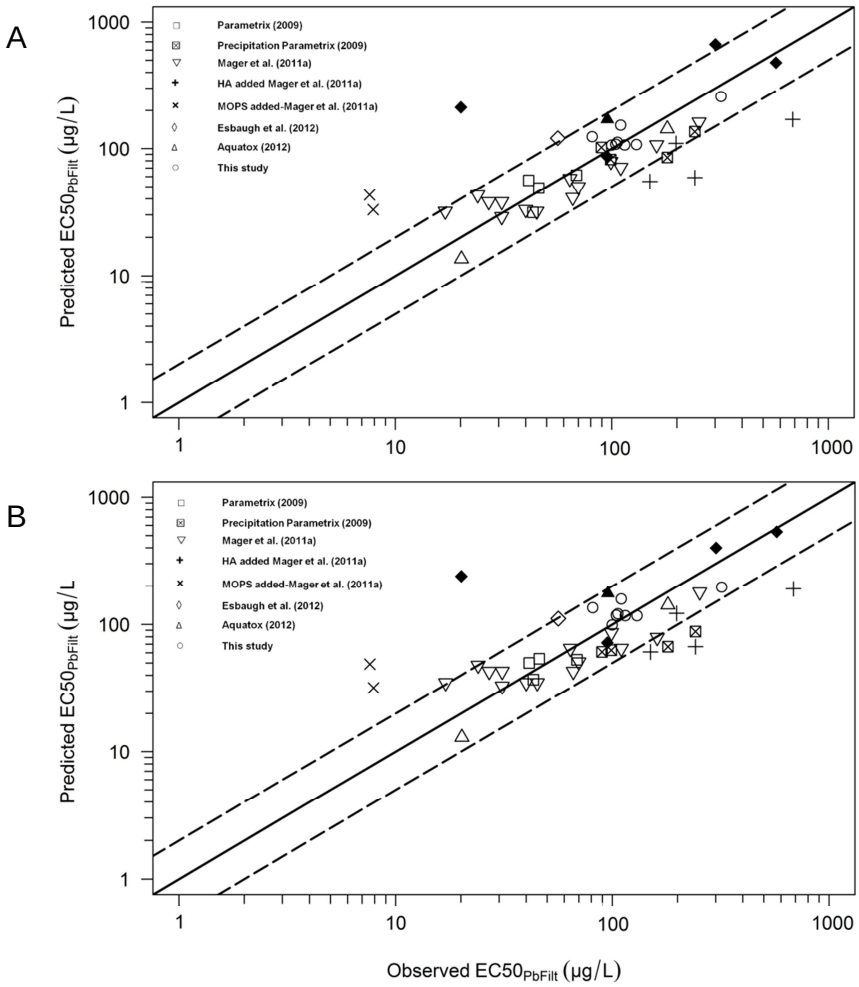

Figure 2.3. Predicted versus observed Pb toxicity to *Ceriodaphnia dubia,* expressed as EC50 (µg filtered Pb/L; based on reproductive toxicity) for the developed BLM calibrated with the overall intrinsic sensitivity (A) and the developed BLM calibrated with the clone-specific intrinsic sensitivities (B): predictions for the data used for the BLM development and the validation. Predictions were made using Equation 4 linked to Visual Minteq. The dashed line represents a difference of a factor of two between the observed and predicted data. The full line represents a perfect fit between observed and predicted data. Open datapoints are from synthetic mediums, filled from natural waters. Crossed symbols represent data points where precipitation is predicted by speciation calculations. The symbols are designated as follows: □ Parametrix (2010), ⊠ waters where precipitation was predicted by Visual Minteq (Parametrix 2010), ▽ Mager et al. (2011a), + HA added media (Mager et al. 2011a), x MOPS added media (Mager et al. 2011a), ◊ Esbaugh et al. (2012), ∆ AquaTox (2012)], ○ data from the present study. Individual test results discussed in the text are: FL1 – French Lake 1 (Esbaugh et al. 2012) FL2 – French Lake 2 (AquaTox 2012), SW – Sweetwater Strand (Esbaugh et al. 2012).

$EC50_{Pbfilt}$ and $EC20_{Pbfilt}$ in media where MOPS buffer or HA was added were mostly overestimated and underestimated with more than twofold error, respectively. When these synthetic media were

excluded mean prediction errors using the overall intrinsic sensitivity were 1.72 and 1.79 for the $EC50_{Pbfilt}$ and $EC20_{Pbfilt}$, respectively. The mean prediction error for the same waters using the clone-specific intrinsic sensitivities were 1.44 and 1.48 for the $EC50_{Pbfilt}$ and $EC20_{Pbfilt}$, respectively. Colloidal Pb precipitation of hydrocerrusite and lead hydroxide was predicted by Minteq in four waters, all of which were from the Parametrix dataset (2010). Lead toxicity was almost always overestimated in these four waters, but was mostly within 2-fold error. The 7d-$EC50_{Pbfilt}$ and 7d-$EC20_{Pbfilt}$ of the five field waters (Esbaugh et al. 2012; AquaTox 2012) were predicted using the overall intrinsic sensitivity with a mean prediction error of 3.83 and 4.37, respectively. Using the clone-specific intrinsic sensitivity, the 7d-$EC50_{Pbfilt}$ and 7d-$EC20_{Pbfilt}$ of the five field waters were predicted with a mean prediction error of 3.37 and 3.65, respectively. However, toxicity in the French Lake 1 field water from the Esbaugh et al. (2012) dataset was underestimated by at least 10 fold. When this water was excluded, 7d-$EC50_{Pbfilt}$ were predicted with an average prediction error of 1.72 and 1.57 using the mean and clone-specific intrinsic sensitivity, respectively.

2.4 DISCUSSION

2.4.1 Effects of Ca and pH on Pb toxicity

While two studies had previously investigated the effects of water chemistry on chronic Pb toxicity to *C. dubia* (Parametrix 2010; Mager et al. 2011a), our study refines the roles of Ca and pH on chronic Pb toxicity to *C. dubia,* obtained using tests performed simultaneously and completely in parallel.

Although the control reproduction in the Ca test series (14 juveniles/female) was slightly lower than required for test validity (15 juveniles/female) according to the USEPA (2002a), effect concentrations were highly reproducible. Indeed, the 1 mM Ca treatment in the Ca series and the pH 7 treatment in the pH series are highly comparable in terms of chemical composition and also in terms of their measured $EC50_{Pbfilt}$ (i.e. 104 and 106 µg filtered Pb/L, respectively). A log-logistic concentration response curve could not be fitted to the 1.0 and 2.5 mM Ca treatment, presumably due to the steep concentration response, i.e. reproduction going from (almost) 100% to (almost) 0% in a narrow concentration interval (i.e. 50 µg/L). Therefore

$EC50_{Pbfilt}$ for these treatments were calculated with a linear regression function. As mentioned above, $EC50_{Pbfilt}$ of the 1.0 mM Ca treatment and the pH 7 treatment, which were highly comparable in chemical composition, were almost identical. Therefore, it is reasonable to assume that the EC50 calculated by linear regression for this test are reliable.

Observed $EC50_{Pbfilt}$ in the present study ranged 4-fold between 81 and 320 µg/L (Table 2.2). The highest toxicity was observed at the lowest Ca concentration, while the lowest toxicity was observed at the highest pH level. A 1.3-1.6-fold lower $EC50_{Pbfilt}$ was observed at the lowest Ca concentration, when compared to the other Ca treatments. This observation appears to be in contradiction with Mager et al. (2011a; 2001b), who found no evidence of a protective Ca effect on chronic or acute Pb toxicity to *C. dubia*. Parametrix (2010) on the contrary reported that an increase in Ca reduced chronic Pb toxicity to *C. dubia*. However, increased Ca concentrations in their study were accompanied with correlated increases of pH and alkalinity. In natural waters, Ca would seldom increase without covariations in among others alkalinity and pH. However, the concurrent increases in Ca and pH hampered unambiguous conclusions about the effects of Ca based on the Parametrix study (2010). The higher toxicity at low Ca concentration observed in our study may suggest a potential competitive effect of Ca on the Pb biotic ligand site. However, a second possible explanation of this observation is that the combination of both low Ca and Pb stress invoked a higher Pb sensitivity than at higher Ca concentrations in our study. It has indeed been shown that some daphnid species have high Ca requirements to support their regular moulting (Hessen & Rukke 2000). Despite the absence of an effect on control reproduction, the *C. dubia* from the 0.25 mM Ca treatment did indeed appear to be more stressed than others, based on the higher percentage of males observed in the control (Table 2.2). In contrast, no such signals of low Ca stress were observed in the Ca test series of Mager et al. (2011a). Regardless of what the explanation is for the observed effect of increasing Ca in our study, the magnitude of the effect is relatively small compared to effects reported earlier for fish (Grosell et al. 2006a; Mager et al. 2011b).

Ca generally has a protective effect against Pb induced mortality to fish during acute (96 h) and chronic (30 d) exposure (Grosell et al. 2006a; Mager et al. 2011b), although mortality occurred mainly during the first week of the chronic exposure (Grosell et al. 2006a). Lead is commonly regarded as a Ca analogue (Verity 1990). Previous research demonstrated that at high Pb concentrations Pb uptake in fish is linked with the Ca^{2+} transport mechanism at the cell

surface (Rogers & Wood 2004). An increase in Ca concentration results in stronger competition between Pb^{2+} and Ca^{2+} for uptake, which could explain the lower toxicity of Pb. However, at low Pb concentrations more relevant for the chronically very sensitive species *C. dubia*, this competitive uptake mechanism may be less relevant (Mager et al. 2011b). Furthermore, Ca did not protect against Pb accumulation and transcriptional responses in *Pimephales promelas* (fathead minnow) during chronic exposure at similar low Pb concentrations (Mager et al. 2008). Uptake of Pb^{2+} under these conditions may instead occur by mimicry of a different ion through a channel or transporter with low Ca^{2+} affinity and high Pb^{2+} affinity (Mager et al. 2011b), although this requires further experimental validation.

In agreement with previous research (Mager et al. 2011a; Parametrix 2010), high pH was found to have a strong protective effect against the chronic Pb toxicity to *C. dubia*. Parametrix (2010) observed in a pH interval from 7 to 8.5 a similar, but less pronounced pH effect. However, they also reported a protective effect at pH 6. Mager et al. (2011a) found higher Pb toxicity at low pH, but noted that their results were probably confounded by the use of MOPS buffer in the test solutions, which resulted in an increase in Pb sensitivity compared to tests which were not MOPS buffered. More recent studies have demonstrated that MOPS and CO_2 atmospheres are inappropriate methods of pH adjustment in Pb toxicity testing (Esbaugh et aL. 2013). Lead toxicity expressed as filtered Pb may be influenced by pH in two ways. First, H^+ ions can compete with Pb^{2+} for binding sites at the biotic ligand, an effect of increasing importance at lower pH (Macdonald et aL. 2002). Second, at high pH, the complexation of Pb^{2+} with OH^-, CO_3^{2-} and DOC becomes increasingly important, resulting in a higher fraction of Pb-complexes, which are less bioavailable than the free Pb^{2+} ion and therefore also less toxic (Turner et al. 1981).

2.4.2 BLM development and validation

The developed Pb BLM for *C. dubia* only included a H^+ competition effect. Ca, Mg and Na were earlier shown not to affect chronic Pb toxicity to *C. dubia* (Mager et al. 2011a). Additional reasons for not including a competitive effect of Ca^{2+}, based on the current study, are discussed further on. The possible influence of DOC and other ligands (eg. CO_3^{2-}, Cl^-, SO_4^{2-}, HCO_3^-) on Pb toxicity was incorporated into the BLM through Pb-complexation effects modeled in Visual Minteq. The effect of pH was modeled in the current Pb BLM as a classic linear H^+

competition term, while in the preliminary *C. dubia* BLM developed based on the Parametrix dataset (2010) a log-linear pH function was used (Esbaugh et al. 2012). Our choice for a linear H^+ competition term was based on the fact that the linear relation between log($EC50_{Pb2+}$) and pH (r=0.90,p=0.10, n=4, regression not shown, S_{pH}=0.59) was not better than the linear relation between $EC50_{Pb2+}$ and H^+ (r=0.99, p=0.003, n=4, Figure 2.2.B). Although, it could be argued that the regression (and the resulting log K_{HBL}) in Figure 2.2.B could have been influenced by the data point at the lowest pH level (highest H^+ activity), the linear regression based on data with only the three lowest H^+ activities (r=0.95, p=0.20, regression not shown) yielded an almost identical log K_{HBL} value (i.e. 7.53) compared to the one based on data from all four pH levels (i.e. 7.59). Thus, based on our dataset, we saw no reason to deviate from the classic BLM concept to model the effect of pH on toxicity of the Pb^{2+} ion. In addition, the Log K_{HBL} based on the same Parametrix dataset (2010) (from which the preliminary BLM was developed (Esbaugh et al. 2012) was 7.50, which is close to the log K_{HBL} of the present study (i.e. 7.59) and clearly within the 95% confidence limits (6.49-7.87). The log K_{HBL} of the *C. dubia* Pb BLM is more than 3 log units higher than the log K_{HBL} of the acute fish BLM, i.e. 4 (Macdonald et al. 2002), which suggests that the H^+ effect is more important for chronic Pb toxicity to *C. dubia* than for acute Pb toxicity to fish.

The mathematical approach for estimating BLM parameters requires assuming univariate changes in Ca or pH, altering one ion while keeping all other water chemistry parameters constant. However, this is in practice not feasible as some chemical elements will always covary when modifying a single physicochemical characteristic. For example, in our Ca series chloride increased concurrently with Ca by about 4.5 mM, while in our pH series alkalinity increased concurrently with pH. However, the alkalinity observed in our test series had probably negligible effects on *C. dubia* reproduction (Cowgill & Milazzo 1991). Furthermore, Mager et al. (2011a) showed that the addition of Cl to test solutions did not influence Pb toxicity. Therefore, we believe that the concurrent changes in Cl and alkalinity in our Ca and pH series had no significant effects on the test outcomes.

The BLM was validated with a set of independent data from four different studies (Parametrix 2010; Mager et al. 2011a; Esbaugh et al. 2012; AquaTox 2012). In total, 36 waters were incorporated in the independent validation, including 5 natural waters. Using a single overall intrinsic sensitivity for all datasets, Pb toxicity, expressed as $EC50_{Pbfilt}$, was predicted within 2-fold

error for 58% of the synthetic test waters. Calibration of the intrinsic sensitivity for each separate *C. dubia* clone, leads to clear improvements in the model predictions. Using the clone-specific intrinsic sensitivity, $EC50_{Pbfilt}$ were predicted within twofold error for 77% of the synthetic waters. The clone-specific intrinsic sensitivities differed up to 3-fold between the 4 independent *C. dubia* clones (i.e. 0.95 to 3.06 nmol/L). The intrinsic sensitivity parameter corresponds to the sensitivity which is independent of water chemistry (De Schamphelaere & Janssen 2002) and should therefore be similar between different studies. However, the differences in sensitivities seem to be in the normal range, since toxicant sensitivities can vary substantially between different lab clones (Baird et al. 1989; 1991). For example, acute Cd toxicity to *D. magna* has been shown to differ up to two orders of magnitude between different lab clones (Baird et al. 1991). Furthermore, variability in pretreatment culturing and/or differences in testing conditions can result in different sensitivities Baird et al. 1989). Additionally, sensitivity within a lab clone can shift with time up to one-order of magnitude within less than 10 generations (Baird & Barata 1997). Some prominent under- and over-estimations can be noted from Figure 2.3. Toxicity in synthetic media where MOPS was used to buffer the pH was underestimated by at least fourfold- a finding in agreement with recent reports that MOPS may not always be suited for pH manipulations in toxicity testing as it might increases the physiological stress by influencing the local pH around ion-uptake sites (Esbaugh et al. 2013). However, this was previously not observed for Cu and Zn toxicity (De Schamphelaere et al. 2004). In addition, $EC50_{Pbfilt}$ in three out of four media where Aldrich Humic Acid (AHA) was used as a source of dissolved organic matter were underestimated by more than twofold. This overestimation of Pb toxicity in media with added AHA may be explained by an underestimation by the NICA-Donnan model in Visual Minteq of the Pb-binding capacity or -binding strength to AHA. The ability of DOC to bind metal ions is dependent on the nature of the DOC, with humic acid generally providing more protection against metal toxicity than fulvic acid (Wood et al. 2011). When media containing MOPS or HA were excluded, Pb toxicity, expressed as $EC50_{Pbfilt}$, was predicted within twofold of those observed for 68% and 92% of the synthetic test waters using the mean- and clone-specific intrinsic sensitivity, respectively. $EC50_{Pbfilt}$ were slightly better predicted than $EC20_{Pbfilt}$. However differences in prediction errors were small.

A Ca effect was not included in the final chronic Pb BLM because of a combination of several reasons. First, Mager et al. (2011a) found no protective effect of Ca on chronic Pb toxicity to *C.*

dubia and the finding that chronic Pb toxicity was slightly higher in our study at the lowest Ca concentration was somewhat uncertain due to a high number of males recorded in this specific test. Second, the effect of Ca^{2+} on Pb^{2+} toxicity was of less importance than the effect of pH. Only a 3-fold range of $EC50_{Pb2+}$ was observed between the lowest and the highest Ca concentration (Figure 2.2.A), while a 13-fold range in $EC50_{Pb2+}$ was observed between the lowest and highest pH (Figure 2.2.B). Third, including a Ca-effect in the BLM did not improve the overall predictive capacity of the BLM. The BLM combining both a single-site H^+- (log K_{HBL}= 7.6) and a single-site Ca^{2+}-competition effect (log K_{CaBL}=3.1, based on the regression in Figure 2.2.A) and the BLM with only a single-site H^+-competition effect (log K_{HBL}= 7.6), both using clone-specific intrinsic sensitivities, predicted Pb toxicity in all synthetic and natural waters (except the test waters containing MOPS or HA and the French Lake 1 test water) with an average prediction error of 1.42 and 1.40-fold, respectively, and with maximum prediction errors of 3.31 and 2.22-fold, respectively. Both models predicted Pb toxicity for 92% of these test waters within 2-fold error (Appendix A; Table A).

Figure 2.4 shows how well the BLM, calibrated with the clone-specific intrinsic sensitivities, predicts the observed Pb toxicity, expressed as $EC50_{Pbfilt}$, in the Parametrix (2010), Mager et al. (2011a) and in the present study. The individual effect of pH on Pb toxicity tested in the present study and in the Parametrix study (2010) (Figure 2.4.A) and the univariate effect of NOM addition (Figure 2.4.B) on Pb toxicity reported by Mager et al. (2011a) were all accurately predicted by the BLM. Ca was univariately varied by Mager et al (2011a) and in our study. The absence of an effect of Ca on Pb toxicity in both those Ca test series was accurately predicted by the developed BLM (Figure 2.4.C). In contrast, Parametrix (2010) reported that increased ambient Ca reduced chronic Pb toxicity to *C. dubia*. However, it should be noted that the Ca effect in that study was not tested in a univariate manner as Ca concentrations co-varied with concentrations of other cations, pH, alkalinity and sulphate. Despite the absence of a Ca effect parameter in the final BLM, the reported trend of increasing $EC50_{Pbfilt}$ with increasing Ca is predicted reasonably well by the BLM (Figure 2.4.D), suggesting that the trend of decreasing toxicity with increasing Ca in this particular study is explained by the co-variation of Ca with other chemical parameters, and not by Ca itself. Colloidal Pb precipitation was predicted by Visual Minteq in four waters, all of which were from the Parametrix dataset. Chronic 7d-Pb toxicity was almost always overestimated in these four waters. Predicted $EC50_{Pbfilt}$ were almost

always closer to the calculated true dissolved concentration than to the observed filtered concentration (Figure 2.4.C & D). This suggests that colloidal Pb precipitation indeed may be present in these waters. Taking into account Pb precipitation in speciation calculations can therefore provide more insight in the role that colloidal Pb mineral formation may play in the observed variation of measured filtered EC50s among test media.

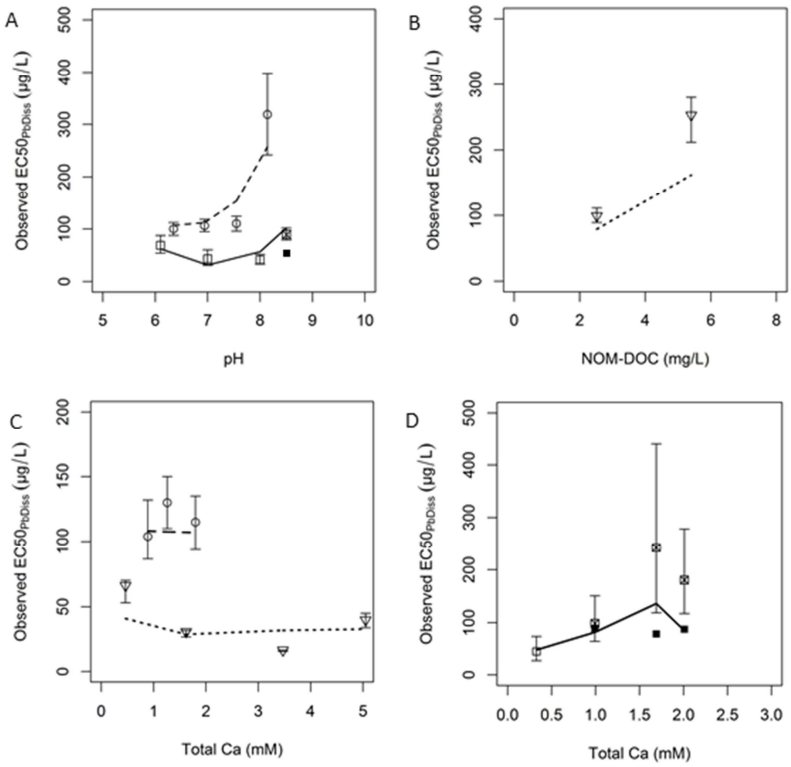

Figure 2.4. Observed (symbols) and predicted (lines) effects of pH (data from the present study and Parametrix (2010); A), DOC (data from Mager et al. (2011a); B), Ca (data from the present study and from Mager et al. (2011a); C), Ca (data from Parametrix (2010); D) on chronic Pb reproductive toxicity to *Ceriodaphnia dubia*. Error bars are 95% confidence limits. The symbols are designated as follows: ○ data from the present study, □ Parametrix (2010), ▽ Mager et al. (2011a). Crossed symbols represent toxicity tests where colloidal Pb precipitation is predicted by Visual Minteq. Filled symbols show truly dissolved Pb concentrations as calculated by Visual Minteq, when colloidal Pb precipitation is predicted. The lines are designated as follows: full line Parametrix (2010), dashed line the present study and dotted line Mager et al. (2011a). The data of the 0.25 mM Ca treatment of the present study is not included for reasons mentioned in the text.

The BLM optimized with the clone-specific intrinsic sensitivity predicted $EC50_{Pbfilt}$ for all field waters within twofold error, except for the French Lake 1 water and the Sweetwater strand water

where toxicity was underestimated with 11-and 2.2-fold error, respectively (Figure 2.3.B). However, the underestimation of toxicity in the French Lake 1 field water was even higher (12-fold) with a preliminary chronic Pb BLM for *C. dubia* (Esbaugh et al. 2012) (Figure A.1 in Appendix A). Esbaugh et al. (2012) argued that the underestimation of Pb toxicity in the French Lake 1 water was potentially caused by the nature of the DOC in this field water, which has low protective capacities, and was not taken into account in the BLM structure. Interestingly, retesting of the same French Lake natural water by AquaTox (2012) but with a sample taken at a later date (i.e. French Lake 2) resulted in an $EC50_{Pbfilt}$ which could be predicted within a factor two error (Figure 2.3.B), suggesting that the French Lake 1 test is likely an outlier for unknown reasons. While the original observed variation in $EC50_{Pbfilt}$ among the natural waters, not considering the French Lake 1 test, was sixfold (i.e. 94.8-573 µg/L), most of the $EC50_{filt}$ were predicted with the new BLM within an error of twofold (Figure 2.3). This suggests that the *C. dubia* BLM presented herein can be used to predict Pb toxicity in natural waters.

To evaluate the improvement of the newly developed BLM (this study) compared to the preliminary BLM (Esbaugh et al. 2012) Pb toxicity, expressed as $EC50_{Pbfilt}$, was predicted for all waters using the same procedure as for the validation of the newly developed BLM with the clone-specific intrinsic sensitivity (see materials and methods), but using the BLM equation described in Esbaugh et al. (2012) instead of Equation 2.4. Overall, the BLM developed in the present study improved predictions of Pb toxicity in the synthetic and natural media when compared to the preliminary BLM developed by Esbaugh et al. (2012) (Appendix A, Figure A.1). When the media where MOPS or HA was added and the French Lake 1 field water were not considered, Pb toxicity was predicted by the preliminary BLM with an average of 1.53 (range=1.01-2.74) and within twofold error for 86% of the waters, while the BLM developed in the present study predicted Pb toxicity with an average of 1.40 (range=1.04-2.22) and for 92% of the synthetic and natural waters within 2-fold error (Appendix A, Table A.5).

2.4.3 Implications for risk assessment

Our results with Pb add to the growing evidence that water hardness is not always the main water chemistry parameter influencing chronic metal toxicity (De Schamphelaere et al. 2004a; Markich et al. 2005; Mager et al. 2011a). This is worrying since water quality criteria for several

metals, including Pb, are still commonly derived using hardness correction equations (e.g. USEPA 2002b; CCME 2013), allowing a higher Pb concentration in surface waters with higher hardness. For example, for a water hardness of 100 mg $CaCO_3$/L, the Canadian water quality guideline for the protection of aquatic life is 3.18 µg filtered Pb/L (CCME 2013), which is close to the 30d-EC20 of ≤4 µg dissolved Pb/L reported for the most sensitive freshwater organism, *Lymnaea stagnalis,* at this hardness level (Grosell et al. 2006b). This raises questions if this and other sensitive species are adequately protected by hardness-based water quality guidelines, especially at high hardness and low DOC concentrations (Markich et al. 2005). BLMs on the contrary have proven their usefulness in predicting chronic metal toxicity in natural waters based on a more complete knowledge of bioavailability-influencing water characteristics (such as pH, DOC, Ca, Mg, Na) (e.g. (De Schamphelaere & Janssen 2004; Deleebeeck et al. 2008)). Therefore, BLM-based water quality criteria are gaining increased attention in the regulatory field. In 2007, BLM-based water quality criteria for Cu were incorporated in the ambient WQC guidelines of the United States (USEPA 2007). Around the same time BLMs were also incorporated in the risk assessment of Cu, Ni and Zn in the European Union (DEPA 2008; ECI 2008; Van Sprang et al. 2009). The BLM-based Cu water quality criteria were already shown to be an improvement compared to hardness-based criteria, especially in waters with high hardness levels (Van Genderen et al. 2007; Meyer & Adams 2010). In addition, BLM-based approaches to calculate freshwater quality criteria for Zn have been developed recently (Van Sprang et al. 2009; DeForest et al. 2012). This illustrates that the incorporation of bioavailability of metals in current water quality guidelines and risk assessments is indispensable and further research is needed to further develop Pb BLMs (also with other organisms) and to investigate how these can be implemented in the risk assessments process. In the meantime, based on the present research with *C. dubia,* BLM-based criteria are likely to be more appropriate relative to hardness-based criteria to address the risk of Pb in surface waters.

3

Calibration of the chronic daphnid Ni, Zn, and Pb bioavailability models in WHAM VII

3. Calibration of the chronic daphnid Ni, Zn, and Pb bioavailability models in WHAM VII

3.1 Introduction

Chronic daphnid bioavailability models exist for Zn (*Daphnia magna*; Heijerick et al. 2005), Ni (*D. magna* and *Ceriodaphnia dubia*; Deleebeeck et al. 2008 and De Schamphelaere et al. 2006, respectively), and Pb (*C. dubia*; Chapter 2). Metal toxicity in these models is typically related to the concentration of metal binding to a biotic ligand, a receptor at the cell surface, and the protective effects of certain cations, such as Ca^{2+}, Mg^{2+}, H^{+}, and Na^{+}, which compete with the metal for biotic ligand binding sites. A chemical speciation model describes the equilibrium speciation in the considered water. However, the above mentioned bioavailability models were developed using different speciation programs: WHAM V (Zn), WHAM VI (Ni), and Visual Minteq 3.0 (Pb). Furthermore, these models also make different assumptions regarding the organic and inorganic ligand complexation.

To model organic ligand complexation, it is generally assumed that dissolved organic matter (DOM) consists of a fraction of active fulvic acid (%AFA) for ion binding, with the other DOM fraction being inert for ion binding (e.g. Cheng et al. 2005). For Ni, it was shown that a %AFA of 40% in combination with a modified binding strength of Ni to FA in WHAM VI (Log $K_{MA(Ni)}$=1.75 instead of the default Log $K_{MA(Ni)}$=1.40) resulted in the best fit between measured and WHAM VI calculated Ni^{2+} activities in a series of spiked natural waters (De Schamphelaere et al. 2006). As a consequence, these Ni-FA binding assumptions were adopted in all chronic Ni bioavailability models (De Schamphelaere et al. 2006; Deleebeeck et al. 2007; 2008; 2009). However, a later study on the same dataset showed that assuming a %AFA of 65% in combination with the default binding strength of Ni to FA in WHAM VI (default Log $K_{MA(Ni)}$=1.40) but increasing the heterogeneity of the FA binding sites ($\Delta LK_{2(Ni\text{-}FA)}$=2.35, instead of default $\Delta LK_{2(Ni\text{-}FA)}$=1.57) resulted in an even better fit between measured and WHAM VI calculated Ni^{2+} activities (Van Laer et al. 2006). The same assumption of a %AFA of 65% has also been used for the chronic *C. dubia* Pb biotic ligand model (BLM) (Chapter 2). For the Zn BLM, it is assumed that 61% of the FA in natural waters is reactive (De Schamphelaere et al. 2005), although other assumptions were used for DOM in synthetic waters (50%AFA) and for DOM originating from specific locations (e.g.

Ankeveen: 71%AFA) (Heijerick et al. 2005). Assumptions of 60% to 70% AFA have been shown to typically work best for predicting metal toxicity in natural waters (Tipping 2002), and the assumption of 65%AFA has been used in several recent metal mixture studies (Lofts & Tipping 2013, 2015).

The use of different speciation programs and varying assumptions between the bioavailability models of Zn, Ni, and Pb currently hinders the integration of these models in a unifying metal mixture bioavailability model to predict toxicity of Zn-Ni-Pb mixtures. Therefore, we evaluated if metal toxicity can be predicted using a single speciation software and a single set of speciation assumptions for all three bioavailability models, without significantly decreasing the predictive power of each of the individual bioavailability models. WHAM VII is the most recent version of the Windermere Humic Aqueous Model. It incorporates the improved Humic-Ion binding model VII (Tipping et al. 2011) and can be regarded as state-of-the-art in speciation modelling. Furthermore, it was shown that free metal ion activity in natural waters could be calculated rather accurately using WHAM VII (Lofts & Tipping 2011). Recently, WHAM VII has also been used to model metal mixture toxicity (Tipping & Lofts 2013; 2015 Iwasaki & Brinkman 2015; Iwasaki et al. 2015). Therefore, we consider WHAM VII as the most appropriate speciation software to model metal speciation. The predictive performance of the bioavailability models coupled with WHAM VII was compared with the predictive performances reported in the original publications (Heijerick et al. 2005; De Schamphelaere 2005; 2006; Deleebeeck et al. 2008; Chapter 2).

The Ni, Zn and Pb bioavailability models calculate inorganic complexation using the stability constants reported by the National Institute of Standards and Technology (NIST; Smith et al. 2004 and Martell et al. 1997, respectively). However, other metal studies have used the default thermodynamic databases in WHAM (e.g. Tipping & Lofts 2013; 2015 Iwasaki & Brinkman 2015; Iwasaki et al. 2015). To assess the importance of the considered inorganic complexation stability constants, we evaluated the impact of the two following speciation scenarios on the prediction capacity of the bioavailability models: I) Using the default thermodynamic database for inorganic complexation; II) Using the stability constants for inorganic complexation reported by NIST (Smith et al. 2004).

3.2 METHODOLOGY

3.2.1 Data selection

The data of the original bioavailability model publications were used to test the validity of the models in WHAM VII. The original data used for the development of the chronic Zn *D. magna* biotic ligand model (BLM) were taken from Heijerick et al. (2005). The 21d-$EC50_{Zndiss}$ and water chemistry parameters were taken from their Table 1. Additionally, the data used for the validation of the chronic Zn BLM in natural waters was taken from De Schamphelaere et al. (2005). The 21d-$EC50_{Zndiss}$ and water chemistry parameters were taken from their Table 5. In total 29 waters (23 synthetic + 6 field waters) were considered for the validation of the chronic Zn BLM.

For Ni, chronic Ni toxicity data of *C. dubia* were taken from De Schamphelaere et al. (2006). Water chemistry parameters were taken from their Annex 3. 10d-$EC50_{Nidiss}$ were taken from their Table 4.16. Chronic Ni toxicity data of *D. magna* was taken from Deleebeeck et al. (2008). Water chemistry parameters were taken from their Table 1 and 2. The corresponding 21d-$EC50_{Nidiss}$ were taken from their Table 3. The Regge field water was not considered as the EC50 in this water could not be accurately estimated.

For Pb, chronic toxicity data for *C. dubia* were taken from Chapter 2, i.e. all Pb toxicity data used in the BLM development (Brisy waters) and those used in the validation (data from Mager et al. (2011), Esbaugh et al. (2012), Parametrix (2010), and Aquatox (2012)) were used. Water chemistry parameters for all toxicity data were taken from Table A.1 in Appendix A. The waters with humic acid additions of the study of Mager et al. (2011) were not considered because it was shown that Pb toxicity in these waters was consistently overestimated (Chapter 2). Additionally, also the waters reported in Mager et al. (2011) which were buffered using 3-(N-morpholino) propanesulfonic acid (MOPS) were not considered since Pb toxicity in these waters was underestimated by at least 4-fold using the chronic *C. dubia* BLM (Chapter 2), indicating that MOPS may not be always suited to manipulate the pH in Pb toxicity tests (Esbaugh et al. 2013). Finally, also the French Lake field water of Esbaugh et al. (2012) was omitted from the evaluation since Pb toxicity in this water was previously been shown to be underestimated with more than 10-fold (Esbaugh et al. 2012; Chapter 2).

3.2.2 Speciation calculations

Chemical speciation in the considered test waters was calculated using WHAM VII (Tipping et al. 2011). MOPS was added to the default solute database (pK_a of 7.2). The default metal - dissolved organic matter (DOM) complexation parameters were used. The original chronic daphnid bioavailability models of Zn, Ni, and Pb are based on different assumptions for the complexation capacity of the dissolved organic matter (DOM). However, assumptions of 60% to 70% reactive FA have been shown to typically work best for predicting metal toxicity in natural waters (Tipping 2002). Therefore, we assumed in the present study that for DOM from natural origin (e.g. field waters) 65% of the DOM is reactive and behaves as isolated fulvic acid (FA; 65% AFA). Additionally; it was assumed that dissolved organic matter (DOM) contains 50% carbon on a weight basis. Accordingly, the measured DOC concentration was multiplied by 1.3 to obtain the FA concentration to be used as the input for speciation calculations. Furthermore, it was assumed that activities of the metal cations Fe^{3+} are controlled by colloidal $Fe(OH)_3$ precipitates using the default equation and solubility product embedded in WHAM VII (Lofts & Tipping 2011). These same assumptions have been used in recent metal studies (Tipping & Lofts 2013; 2015). If measured DOC concentrations were not reported for synthetic waters, it was assumed that these waters contained 0.5 mg DOC/L (USEPA 2007).

Table 3.1: Default and adapted stability constants of Ni, Zn and Pb for inorganic complexes used for speciation calculations in WHAM VII.

Element	Parameter	Default stability constant in WHAM VII (log K)	Adapted stability constant (log K)[a]
Ni	$K = [NiCO_3]/\{[Ni^{2+}].[CO_3^{2-}]\}$	5.78	4.57
	$K = [NiHCO_3^+]/\{[Ni^{2+}].[H^+].[CO_3^{2-}]\}$	13.41	12.42
Zn	$K = [ZnCO_3]/\{[Zn^{2+}].[CO_3^{2-}]\}$	4.76	4.76
	$K = [ZnHCO_3^+]/\{[Zn^{2+}].[H^+].[CO_3^{2-}]\}$	13.12	11.83
	$K = [Zn(OH)_2]/\{[Zn^{2+}].[OH^-].[OH^-]\}$	11.1	10.2
Pb	$K = [PbCO_3]/\{[Pb^{2+}].[CO_3^{2-}]\}$	7.2	6.53
	$K = [PbHCO_3^+]/\{[Pb^{2+}].[H^+].[CO_3^{2-}]\}$	13.23	13.23

[a] As proposed by the National Institute of Standards and Technology (Smith et al. 2004)

Two speciation scenarios were evaluated in the present study. In the first scenario (**'Speciation Scenario I'**), the WHAM VII default parameters for inorganic ligand-metal complexation were used (Table 3.1). In the second scenario (**'Speciation Scenario II'**), the stability constants for inorganic

ligand complexation were adapted to those reported by the National Institute for Standards and Technology (Table 3.1). The calculated metal speciation under both scenarios was used to predict individual chronic Zn, Ni and Pb toxicity to daphnids using the metal-specific bioavailability models (see Section 3.2.3). The predictive performance of the bioavailability models under both speciation scenarios was evaluated by comparing bioavailability-model predicted $EC50_{Mediss}$ with observed $EC50_{Mediss}$.

3.2.3 Bioavailability modelling

3.2.3.1 Zn bioavailability modelling

Zn^{2+} toxicity was predicted using Equation 3.1 (Heijerick et al. 2005):

$$EC50_{Zn^{2+},i} = EC50^{*}_{Zn^{2+}}\left(1 + K_{CaBL_{Zn}}\{Ca^{2+}\}_i + K_{MgBL_{Zn}}\{Mg^{2+}\}_i + K_{NaBL_{Zn}}\{Na^{+}\}_i + K_{HBL_{Zn}}\{H^{+}\}_i\right) \quad (3.1)$$

In Equation 3.1, $EC50_{Zn2+,i}$ is the predicted 50% effective concentration of Zn^{2+} in test solution *i*. $K_{CaBL,Zn}$, $K_{MgBL,Zn}$, K_{NaBLZn} and $K_{HBL,Zn}$ are the stability constants for binding of Ca^{2+}, Mg^{2+}, Na^{+} and H^{+} to the Zn biotic ligand, respectively (Table 3.2). $\{Mg^{2+}\}_i$, $\{Ca^{2+}\}_i$, $\{Na^{+}\}_i$, and $\{H^{+}\}_i$ are the chemical activities of Mg^{2+}, Ca^{2+}, Na^{+} and H^{+} in test solution *i* (mol/L). $EC50^{*}_{Zn2+}$ is the intrinsic sensitivities of the Zn BLM, which can be regarded as the $EC50_{Zn2+}$ of daphnid in a solution were all cationic competition effects are absent (Heijerick et al. 2005). The intrinsic sensitivity $EC50^{*}_{Zn2+}$ can be calculated from the observed EC50 using Equation 3.2:

$$EC50^{*}_{Zn^{2+}} = \prod_i^n \left(\frac{EC50_{Zn^{2+},i,observed}}{1+K_{CaBL_{Zn}}\{Ca^{2+}\}_i+K_{MgBL_{Zn}}\{Mg^{2+}\}_i+K_{NaBL_{Zn}}\{Na^{+}\}_i+K_{HBL_{Zn}}\{H^{+}\}_i}\right)^{\frac{1}{n}} \quad (3.2)$$

In this equation, *n* is the number of test solutions considered. $EC50_{Zn2+,i,observed}$ is the observed Zn^{2+} activity in test solution *i* (mol/L). A dataset-specific intrinsic sensitivity was calculated for the data of Heijerick et al. (2005) and De Schamphelaere et al. (2005) separatly. However, the 4 mM Ca treatment of Heijerick et al. (2005) was not included in the intrinsic sensitivity calculations due to the Ca toxicity observed in this treatment (Heijerick et al. 2005). Additionally, also the Rhine and Voyon field water of De Schamphelaere et al. (2005) were excluded from the intrinsic sensitivity calculations since the Zn *D. magna* BLM has shown to be only valid up to pH

8 (De Schamphelaere et al. 2005). The predicted $EC50_{Zn2+,i}$ were then transformed to $EC50_{Zndiss,i,pred}$ using WHAM VII and compared to $EC50_{Zndiss,i,observed}$.

Table 3.2: Model parameters of the chronic Zn BLM for *Daphnia magna*[1], the chronic Ni bioavailability model for *D. magna*[2] and *Ceriodaphnia dubia*[3], and the chronic Pb BLM for *C. dubia*[4]

	Zn *D. magna* BLM[1]	Ni *D. magna* model[2]	Ni *C. dubia* model[3]	Pb *C. dubia* BLM[4]
Log K_{MgBL}	2.69	3.57	3.57	-
Log K_{CaBL}	3.22	3.53	3.53	-
Log K_{NaBL}	1.90	-	-	-
Log K_{HBL}	5.77	-	-	7.6
S_{pH}	-	0.3335	0.8587	-

[1] Heijerick et al. (2005); Equation 3.1
[2] Deleebeeck et al. (2008): Equation 3.3
[3] De Schamphelaere et al. (2006); Equation 2.3
[4] Chapter 2; Equation 3.5

3.2.3.2 Ni bioavailability modelling

Ni^{2+} toxicity to *D. magna* and *C. dubia* was predicted using the bioavailability model reported by Deleebeeck et al. (2008) and De Schamphelaere et al. (2006). Both bioavailability models have the same model structure (Eq. 3.3), but differ in the magnitude of the slope of the effect of pH on Ni^{2+} toxicity ($S_{pH,Ni}$).

$$EC50_{Ni^{2+},i} = 10^{-(S_{pH_{Ni}} \cdot pH_i + Q50_{Ni})}\left(1 + K_{CaBL_{Ni}}\{Ca^{2+}\}_i + K_{MgBL_{Ni}}\{Mg^{2+}\}_i\right) \quad (3.3)$$

In Equation 3.3, , $EC50_{Ni2+,i}$ is the predicted 50% effective concentration of Ni^{2+} in test solution *i*. $Q50_{Ni}$ is the intrinsic sensitivity of the chronic Ni bioavailability model. The intrinsic sensitivity of the Ni bioavailability model is the intercept of the linear relationship between log $EC50_{Ni2+}$ and pH, after correction for Ca^{2+} and Mg^{2+} competition (Deleebeeck et al. 2008; De Schamphelaere et al. 2006). $K_{CaBL,Ni}$ and $K_{MgBL,Ni}$ are the stability constants for binding of Ca^{2+} and Mg^{2+} to the Ni biotic ligand, respectively. $S_{pH,Ni}$ is the pH slope of Ni toxicity in the Ni bioavailability models (S_{pH}=0.8587 for *C. dubia* and S_{pH}=0.3335 for *D. magna*) and pH_i is the pH of test solution *i*. { Mg^{2+}}, and $\{Ca^{2+}\}_i$ are the chemical activities of Mg^{2+}, and Ca^{2+} in test solution *i* (mol/L), respectively. All model parameters are listed in Table 3.2.

The intrinsic sensitivity, Qx_{Ni2+}, were calculated from the observed ECx_{Ni2+} using Equation 3.4.

$$Q50_{Ni^{2+}} = \frac{\sum_i^n \left(-log\left(\frac{EC50_{Ni^{2+},i,observed}}{1+K_{CaBL_{Ni}}\{Ca^{2+}\}_i + K_{MgBL_{Ni}}\{Mg^{2+}\}_i} \right) - S_{pH_{Ni}} \cdot pH_i \right)}{n} \quad (3.4)$$

In these equation, $EC50_{Ni2+,i,observed}$ is the observed Ni^{2+} activity in test solution *i* at the 50% effective concentration (mol/L). The $Q50_{Ni2+}$ of *C. dubia* was calculated based on the observed EC50 of all field waters reported by De Schamphelaere et al. (2005). The $Q50_{Ni2+}$ of *D. magna* was calculated based on the same observed EC50 as used by Deleebeeck et al. (2008), i.e. all synthetic waters, except the pH 8 synthetic water (extrapolated EC50). The predicted $EC50_{Ni2+,i}$ were then translated to $EC50_{Nidiss,pred,i}$ using WHAM VII and compared to observed $EC50_{Nidiss,i}$.

3.2.3.3 Pb bioavailability modelling

Pb^{2+} toxicity to *C. dubia* was predicted using the chronic Pb BLM developed in Chapter 2 (Eq. 3.5):

$$EC50_{Pb^{2+},i} = EC50^*_{Pb^{2+}}\left(1 + K_{HBL_{Pb}}\{H^+\}_i\right) \quad (3.5)$$

In Equation 3.5, $EC50_{Pb2+,i}$ is the predicted 50% effective concentration of Pb^{2+} in test solution *i*. $EC50^*_{Pb2+}$ is the intrinsic sensitivity of the Pb BLM, which can be regarded as the $EC50_{Pb2+}$ of *C. dubia* in a solution were all cationic competition effects are absent (Chapter 2). $K_{HBL,Pb}$ is the stability constants for binding of H^+ to the Pb biotic ligand. $\{H^+\}_i$ is the chemical activity of H^+ in test solution *i* (mol/L). All model parameters are listed in Table 3.2.

Bioavailability modelling starts with calculating the intrinsic sensitivity from the observed $EC50_{Pb2+}$ using Equation 3.6:

$$EC50^*_{Pb^{2+}} = \prod_i^n \left(\frac{EC50_{Pb^{2+},i,observed}}{1+K_{HBL_{Pb}}\{H^+\}_i} \right)^{\frac{1}{n}} \quad (3.6)$$

In equation 3.6, $EC50_{Pb2+,i,observed}$ is the observed Pb^{2+} activity in test solution *i* (mol/L). The intrinsic sensitivity was calculated based on the same waters as in Chapter 2: all waters were considered except for the Ca 1.00 mM, Ca 1.75 mM, Ca 2.25 mM and pH 8.6 of Parametrix (2010) and the 0.25 mM Ca water of the developmental series in Chapter 2. The intrinsic sensitivity is then used as input in Equation 3.5 to predict $EC50_{Pb2+,i}$ for all considered test

solutions. The predicted $EC50_{Pb2+,i}$ are translated to $EC50_{Pbdiss,i}$ using WHAM VII and compared to observed $EC50_{Pbdiss,i}$.

3.3 Results

3.3.1 Validation of the Zn *D. magna* BLM in WHAM VII

The intrinsic sensitivities ($EC50^*_{Zn2+}$) for the Speciation Scenario I and II are reported in Table 3.3. Both speciation scenarios predicted chronic Zn toxicity to *D. magna* with reasonable accuracy (Figure 3.1; Table 3.4), i.e. almost all $EC50_{Zndiss}$ were predicted within 2-fold error. The speciation scenario using the NIST stability constants for inorganic ligand complexation predicted $EC50_{Zndiss}$ slightly more accurately, certainly in the natural water dataset. Using the Speciation Scenario I, Zn toxicity in the Voyon and Rhine field water were underestimated with 3.0 and 2.8-fold error, respectively. These waters are characterized by a high pH (pH 8.2 & 8.4 for Rhine & Voyon, respectively). It has previously been reported that the Zn *D. magna* BLM underestimates Zn toxicity at pH above 8 (De Schamphelaere et al. 2005). However, Zn toxicity in these high pH waters is considerably better predicted using the modified stability constants for inorganic complexation (Figure 3.1).

Overall, the Zn *D. magna* BLM coupled with WHAM VII predicted Zn toxicity in natural and synthetic waters at least as accurate as the original Zn *D. magna* BLM coupled with WHAM V (Table 3.4).

Table 3.3. Average intrinsic Zn^{2+} sensitivities for *Daphnia magna* (21d-EC50*Zn2+; calculated using Eq. 3.2) under Speciation Scenario I and Speciation Scenario II.

	Synthetic waters (nmol/L)[a]	Natural waters (nmol/L)[b]
Speciation scenario I[c]	718	609
Speciation scenario II[d]	746	731

[a] Data from Heijerick et al. (2005)
[b] Data from De Schamphelaere et al. (2005)
[c] Speciation Scenario I: default WHAM VII stability constants for inorganic complexation
[d] Speciation Scenario II: stability constants for inorganic complexation reported by NIST

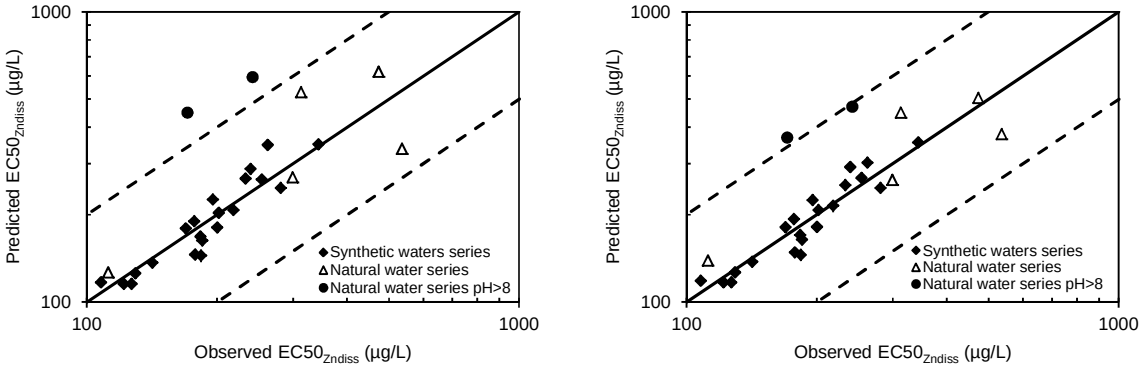

Figure 3.1. Predicted versus observed median effective concentrations ($EC50_{Zndiss}$, reproductive toxicity expressed as µg dissolved Zn/L) for *Daphnia magna* using Speciation Scenario I (i.e. using the default WHAM VII stability constants for inorganic complexation; left) and Speciation Scenario II (i.e. using stability constants for inorganic complexation reported by NIST; right) Predictions were made using the chronic Zn *D. magna* biotic ligand model (Eq. 3.1 linked to WHAM VII). Dashed line represents a difference of a factor of 2 between the observed and predicted data. Full line represents a perfect fit between the observed and predicted data.

Table 3.4. Prediction statistics of 21d-$EC50_{Zndiss}$ predicted with the chronic Zn *Daphnia magna* biotic ligand model (Eq. 3.1) in WHAM V[a,b] and in WHAM VII using Speciation Scenario I and Speciation Scenario II.

	WHAM V		WHAM VII- Speciation scenario I[c]			WHAM VII- Speciation scenario II[d]		
	Synthetic waters[a]	Natural waters[b]	All data	Synthetic waters	Natural waters	All data	Synthetic waters	Natural waters
Mean prediction error	1.22	1.53	1.28	1.15	1.70	1.22	1.14	1.48
Median prediction error	1.18	1.35	1.13	1.09	1.58	1.10	1.09	1.41
Maximum prediction error	1.75	2.09	2.62	1.97	2.62	2.15	1.99	2.15
% predicted within 2-fold error	100	86	93	100	71	97	100	86

[a] As reported in Heijerick et al. (2005); n=22
[b] As reported in De Schamphelaere et al. (2005), n=7
[c] Speciation Scenario I: default WHAM VII stability constants for inorganic complexation
[d] Speciation Scenario II: stability constants for inorganic complexation reported by NIST

3.3.2 Validation of the chronic Ni *C. dubia* bioavailability model

Recalibrated intrinsic sensitivities for *C. dubia* for both speciation scenarios are listed in Table 3.5. Generally, the Speciation Scenario II predicted chronic Ni toxicity to *C. dubia* more accurately than the Speciation Scenario I (Table 3.6; Figure 3.2). Moreover, the predictions of Speciation Scenario I show a bias, i.e. predictions of $EC50_{Nidiss}$ are not parallel along the 1:1 line (Figure 3.2 left panel), while a similar bias was not observed for the Speciation Scenario II. Chronic Ni toxicity to *C. dubia* was slightly less well predicted when the Ni *C. dubia* bioavailability model was coupled to WHAM VII than when it was coupled to WHAM VI (Table 3.6).

Table 3.5. Average intrinsic Ni^{2+} sensitivities for *Ceriodaphnia dubia* using the Ni *C. dubia* model (Eq. 3.4) under Speciation Scenario I and Speciation Scenario II.

	$Q50_{Ni2+}$
Speciation scenario I[b]	1.34
Speciation scenario II[c]	1.00

[a] Data from De Schamphelaere et al. (2006)
[b] Speciation Scenario I: default WHAM VII stability constants for inorganic complexation
[c] Speciation Scenario II: stability constants for inorganic complexation reported by NIST

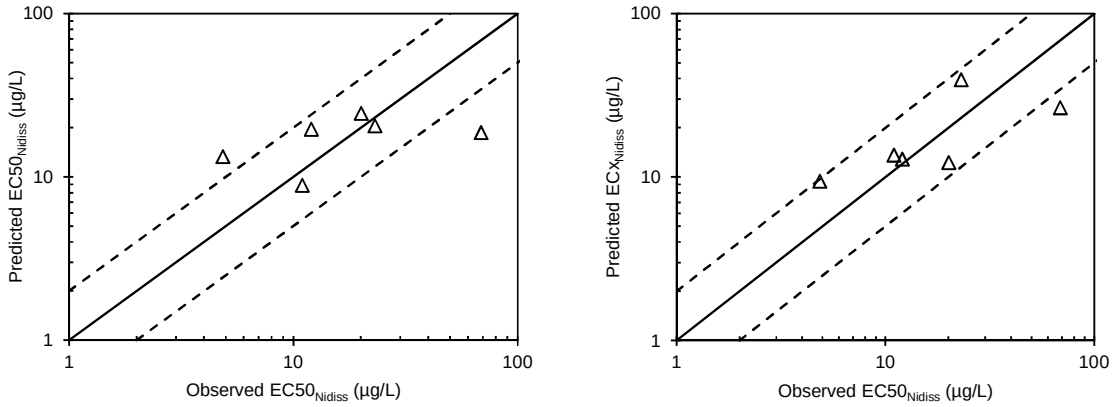

Figure 3.2 Predicted versus observed chronic Ni toxicity to *Ceriodaphnia dubia*, expressed as 50% effective concentrations for reproductive toxicity (10d-$EC50_{Nidiss}$, µg dissolved Ni/L) under Speciation Scenario I (i.e. using the default WHAM VII stability constants for inorganic complexation; left) and Speciation Scenario II (i.e. using stability constants for inorganic complexation reported by NIST; right). Predictions were made using the chronic *C. dubia* Ni bioavailability model (Eq. 3.3) linked to WHAM VII. Dashed line represents a difference of a factor of 2 between the observed and predicted data. Full line represents a perfect fit between the observed and predicted data.

Table 3.6. Prediction statistics of *Ceriodaphnia dubia* 10d-$EC50_{Nidiss}$ predicted with the chronic Ni *C. dubia* bioavailability model (Eq. 3.3) in WHAM VI and WHAM VII using Speciation Scenario I[a] and Speciation Scenario II.[b]

	WHAM VI[a]	WHAM VII-Speciation scenario I[b]	WHAM VII-Speciation scenario II[c]
n	6	6	6
Mean prediction error	1.37	1.94	1.70
Median prediction error	1.19	1.43	1.67
Maximum prediction error	1.96	3.68	2.58
% predicted within 2-fold error	100	67	83

[a] As reported by De Schamphelaere et al. (2006)
[b] Speciation Scenario I: default WHAM VII stability constants for inorganic complexation
[c] Speciation Scenario II: stability constants for inorganic complexation reported by NIST

For *D. magna*, the prediction of Ni toxicity was more complicated. When the *D. magna* Ni bioavailability model was calibrated on the synthetic waters (Table 3.7), Ni toxicity to *D. magna* was more accurately predicted using Speciation Scenario I compared to Speciation Scenario II

(Figure 3.3 filled symbols; Table 3.8). However, predictions of Ni toxicity in the natural waters were biased (most of the filled squares below the 1:1 line in Figure 3.3), certainly for the Speciation Scenario II (Figure 3.3 right panel). Deleebeeck et al. (2008) reported that Ni sensitivity may have been shifted between the synthetic water test series and the natural water test series. Therefore, we recalibrated the intrinsic sensitivity of the *D. magna* bioavailability model specifically on the natural waters (natural waters-specific intrinsic sensitivity; Table 3.7). Using this recalibrated intrinsic sensitivity, the overall prediction error was similar between Speciation Scenario I and Speciation Scenario II (Table 3.9; Figure 3.3 open symbols). However, Ni toxicity to *D. magna* was predicted more accurately under Speciation Scenario II.

Overall, the prediction performance of the *D. magna* bioavailability model under Speciation Scenario II was relatively comparable with those of the performance of the original *D. magna* bioavailability model reported by Deleebeeck et al. (2008) (Table 3.9).

Table 3.7. Average intrinsic Ni^{2+} sensitivities for *Daphnia magna* using the chronic Ni *D. magna* bioavailability model (calculated using Eq. 3.4) under Speciation Scenario I and Speciation Scenario II. Toxicity data originating from Deleebeeck et al. (2008)

	Synthetic water-intrinsic sensitivity [a]	Natural waters-specific intrinsic sensitivity [b]
	$Q50_{Ni2+}$	Natural waters $Q50_{Ni2+}$
Speciation scenario I[c]	4.54	4.27
Speciation scenario II[d]	4.47	3.98

[a] The synthetic water intrinsic sensitivity was calibrated on the synthetic waters (n=17).
[b] The natural waters-specific intrinsic sensitivity was calibrated on the natural waters (n=5) separately.
[c] Speciation Scenario I: default WHAM VII stability constants for inorganic complexation
[d] Speciation Scenario II: stability constants for inorganic complexation reported by NIST

Table 3.8. Prediction statistics of *Daphnia magna* 21d-$EC50_{Nidiss}$ predicted with the chronic Ni *D. magna* bioavailability model (Eq. 3.3) in WHAM VII using Speciation Scenario I and Speciation Scenario II. The model was calibrated on all data (i.e. predictions were made with the overall intrinsic sensitivity)

	WHAM VI[a]		WHAM VII-Speciation Scenario I[b]			WHAM VII-Speciation Scenario II[c]		
	Natural waters	Synthetic waters	All data	Natural waters	Synthetic waters	All data	Natural waters	Synthetic waters
n	5	17	22	5	17	22	5	17
Mean prediction error	1.9	1.3	1.42	2.11	1.22	1.71	3.10	1.30
Median prediction error	-	-	1.15	1.51	1.12	1.34	3.51	1.26
Maximum prediction error	2.6	1.6	4.10	34.10	1.63	3.97	3.97	1.94
% predicted within 2-fold error	60	100	91	60	100	82	20	100

[a] Prediction performance of the *D. magna* Ni bioavailability model coupled with WHAM VI as reported in Deleebeeck et al. (2008)
[b] Speciation Scenario I: default WHAM VII stability constants for inorganic complexation
[c] Speciation Scenario II: stability constants for inorganic complexation reported by NIST

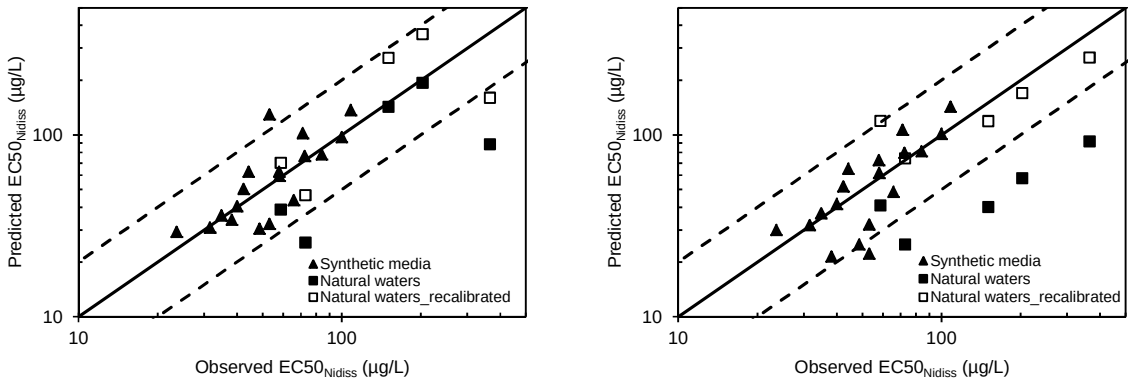

Figure 3.3 Predicted versus observed chronic reproductive Ni toxicity to *Daphnia magna*, expressed as 50% effective concentrations (21d-$EC50_{Nidiss}$, µg dissolved Ni/L) under Speciation Scenario I (i.e. using the default WHAM VII stability constants for inorganic complexation; left) and Speciation Scenario II (i.e. using stability constants for inorganic complexation reported by NIST; right) Predictions were made using the chronic *D. magna* Ni bioavailability model (Eq. 3.3) linked to WHAM VII. Dashed line represents a difference of a factor of 2 between the observed and predicted data. Full line represents a perfect fit between the observed and predicted data. Filled symbols denote the predictions of the model calibrated on the synthetic waters. Open symbols represent the predictions of the model which was recalibrated on the synthetic and natural waters separately.

Table 3.9. Prediction statistics of *Daphnia magna* 21d-$EC50_{Nidiss}$ predicted with the chronic Ni *D. magna* bioavailability model (Eq. 3.3) in WHAM VII using Speciation Scenario I and Speciation Scenario II. The model was calibrated on the natural waters & synthetic waters separately (i.e. predictions were made with the Dataset specific intrinsic sensitivity)

	WHAM VI[a]		WHAM VII-Speciation Scenario I[b]			WHAM VII-Speciation Scenario II[c]		
	Natural waters	Synthetic waters	All data	Natural waters	Synthetic waters	All data	Natural waters	Synthetic waters
n	5	17	22	5	17	22	5	17
Mean prediction error	1.2	1.3	1.33	1.71	1.22	1.32	1.38	1.30
Median prediction error	-	-	1.22	1.76	1.12	1.26	1.27	1.26
Maximum prediction error	1.5	1.6	2.28	2.28	1.63	2.03	2.03	1.94
% predicted within 2-fold error	100	100	95	80	100	95	80	100

[a] Prediction performance of the *D. magna* Ni bioavailability model coupled with WHAM VI as reported in Deleebeeck et al. (2008)
[b] Speciation Scenario I: default WHAM VII stability constants for inorganic complexation
[c] Speciation Scenario II: stability constants for inorganic complexation reported by NIST

3.3.3 Validation of the chronic Pb *C. dubia* BLM

The intrinsic Pb sensitivities of the Speciation Scenario I and Speciation Scenario II were 2.57 and 3.67 nmol/L, respectively. Using these intrinsic sensitivities, the majority of the $EC50_{Pbdiss}$ were predicted within 2-fold error (Figure 3.4; Table 3.10). The predictions capacities of the Pb BLM using these Speciation Scenarios were similar as when the original chemical speciation

model Visual Minteq 3.0 was used (Tabel 3.10). Using either the default stability constant for inorganic metal complexes in WHAM VII (Speciation Scenario I) or those reported by NIST (Speciation Scenario II) did not have a significant effect on the model predictions (Table 3.10). However, the difference between the default and NIST inorganic ligand stability constants were relatively small (i.e. less than an order magnitude), compared to the other metals (Table 3.1).

Table 3.10. Prediction statistics of 8d-EC50$_{Pbdiss}$ (n=32) predicted with the chronic Pb *Ceriodaphnia dubia* BLM (Eq. 3.5) in WHAM VII under the original speciation assumptions, Speciation Scenario I and Speciation Scenario II.

	Visual Minteq[a]	WHAM VII-Speciation scenario I[b]	WHAM VII-Speciation scenario II[c]
Mean prediction error	1.48	1.60	1.63
Median prediction error	1.29	1.52	1.44
Maximum prediction error	2.54	3.50	4.32
% predicted within 2-fold error	78	81	81

[a] Based on the data from Chapter 2
[b] Speciation Scenario I: default WHAM VII stability constants for inorganic complexation
[c] Speciation Scenario II: stability constants for inorganic complexation reported by NIST

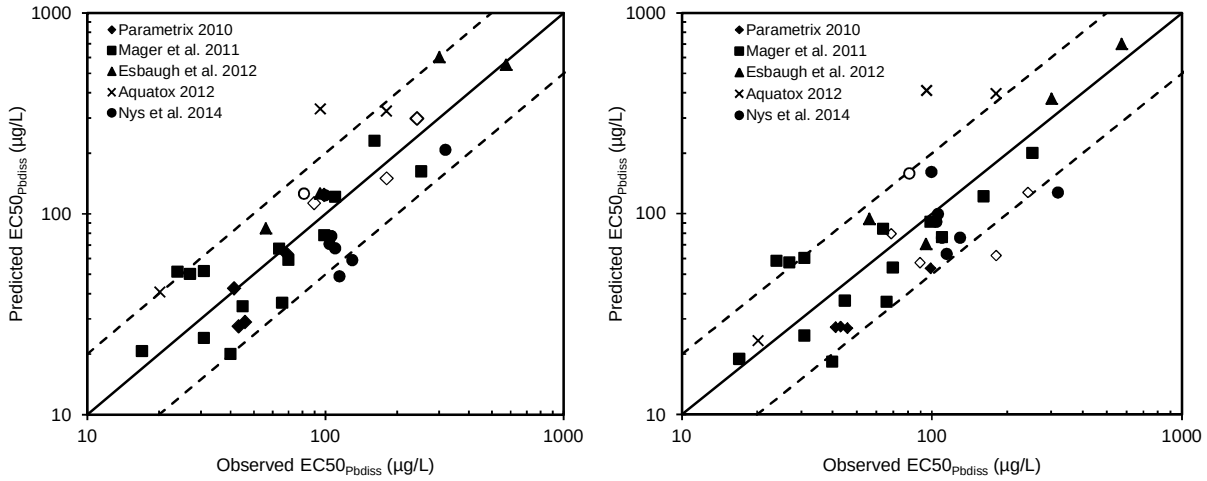

Figure 3.4 Predicted versus observed chronic Pb toxicity to *Ceriodaphnia dubia*, expressed as 50% effective concentrations (8d-EC50$_{Pbdiss}$, µg dissolved Pb/L) under Speciation Scenario I (using the default WHAM VII stability constants for inorganic complexation; left) and Speciation Scenario II (i.e. using stability constants for inorganic complexation reported by NIST; right) Predictions were made using the chronic *C. dubia* Pb BLM (Equation 3.5) linked to WHAM VII. Dashed line represents a difference of a factor of 2 between the observed and predicted data. Full line represents a perfect fit between the observed and predicted data. Open symbols represent the waters which were not considered for intrinsic sensitivity calculations.

3.4 Discussion

Overall, the chronic daphnid bioavailability models for Zn, Ni, and Pb performed relatively well when the models were calibrated on metal speciation calculated with WHAM VII. A bioavailability model is generally accepted to be sufficiently accurate if the majority of $EC50_{Mediss}$ is predicted within 2-fold error (Di Toro et al. 2001; Santore et al. 2001; Heijerick et al. 2005; De Schamphelaere 2005; 2006; Deleebeeck et al. 2008; Chapter 2), this was the case for all bioavailability models. Additionally, the prediction performance in WHAM VII approached those reported in the original publications (Heijerick et al. 2005; De Schamphelaere et al. 2005; 2006; Deleebeeck et al. 2008; Chapter 2). This observation indicates that the daphnid bioavailability models can be used in combination with WHAM VII without the need for reparametrisation of the model parameters, such as the biotic ligand stability constants and/or pH slopes.

The identity of the inorganic thermodynamic database had an impact on the prediction performance of the chronic Ni and Zn daphnid bioavailability models. The prediction performance of the latter models were generally better when the stability constants for inorganic complexation reported by NIST were used, certainly in natural waters. The prediction capacities of the Pb BLM were less impacted.

Lofts & Tipping (2011) reported that WHAM VII (using the default inorganic complexation constants) predicts free Ni^{2+} and Zn^{2+} activities in natural waters relatively accurately. However, WHAM VII was primarily a good predictor of Ni^{2+} activity at high Ni concentrations, while it overestimated Ni^{2+} activity at lower Ni concentrations (Lofts & Tipping 2011). Similar observations were previously made when WHAM VI was used to predict measured Ni^{2+} concentrations activities in natural waters (De Schamphelaere et al. 2006; Van Laer et al. 2006). As a consequence, the influence of the parameters describing Ni-FA complexation on free Ni^{2+} activity calculations were evaluated. De Schamphelaere et al. (2006) observed that increasing the Ni-FA binding constant (log $K_{Ni\text{-}FA}$) and lowering the %AFA resulted in a better fit between measured and WHAM VI calculated Ni^{2+}. Therefore, the default Ni-FA complexation parameters in WHAM VI were adapted in the original Ni bioavailability models (De Schamphelaere et al. 2006; Deleebeeck et al. 2007; 2008; 2009). We observed that Ni toxicity predictions using WHAM VII were relatively accurate, although they were slightly less accurate than those reported by De Schamphelaere et al. (2006) and Deleebeeck et al. (2008). It is possible that this difference in prediction performance is the result of a difference in the modelling of the Ni-FA complexation.

Alternatively, there are several other differences in the speciation assumption between the original Ni bioavailability models and the speciation calculations performed here. For instance, the Ni *C. dubia* bioavailability model takes also into account the competing effects between Ni^{2+} and Zn^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+}, and Pb^{2+} for the DOC binding sites and the competing effects of Fe^{3+} are calculated in a different manner, although these assumptions do not have a major effect on the predictions of the Ni bioavailability models coupled with WHAM VII (data not shown). A more in-depth evaluation of the impact of the WHAM VII FA-Ni complexation parameters on the prediction of Ni^{2+} activities could resolve this issue, but such an evaluation falls outside the scope of the present study. However, the default Ni-FA complexation constants predicted Ni toxicity relatively accurately, and can therefore in the mean-time be used to model Ni^{2+} speciation in metal mixture bioavailability models.

Lofts & Tipping (2011) showed that WHAM VII generally predicted measured Pb^{2+} activity with reasonable accuracy. Our results showed that when using WHAM VII similar prediction capacities as when the original speciation assumptions in Visual Minteq 3.0 were used can be achieved. Therefore, WHAM VII can possibly be used as the chemical speciation model in the Pb BLM, although most chronic Pb bioavailability models have used Visual Minteq 3.0 to model chemical speciation (Esbaugh et al. 2012; De Schamphelaere et al. 2014; Chapter 2). Visual Minteq (KTH) was originally selected to model chemical speciation in the Pb bioavailability models because it is the only available speciation software that allows in a single framework the calculation of the formation of inorganic Pb complexes, complexes of Pb with FA, and precipitation of minerals. Indeed, at low pH most Pb in freshwater solutions will be present as free Pb^{2+} ion, while at pH around neutrality or higher Pb carbonate and hydroxide complexes ($Pb(OH)_2(s)$, cerrusite, and hydrocerrusite) will dominate in the solution (Mager 2012). These complexes may be present in the solution as colloidal precipitates. As a consequence, Pb toxicity, expressed as dissolved Pb concentration (i.e. the Pb concentration passing through a 0.45 µm filter), can be higher than the truly dissolved Pb toxicity, because possible colloidal precipitates of Pb minerals can theoretically pass the filters. The original chemical speciation model of the chronic Pb bioavailability models took into account the possible precipitation of Pb minerals (Esbaugh et al. 2012; De Schamphelaere et al. 2014; Chapter 2), but using WHAM VII this is not possible at the moment.

Overall, our results show that WHAM VII with 65%AFA and default metal-FA binding constants can be used as speciation model to predict metal toxicity in aquatic environments with reasonable accuracy. The stability constants for inorganic complexation reported by NIST describe metal toxicity more accurately than the default WHAM VII inorganic stability constants, especially for Ni and Zn.

4

The effect of pH on chronic Zn toxicity differs between daphnid species: development of a preliminary chronic Zn *C. dubia* bioavailability model

4. The effect of pH on chronic Zn toxicity differs between daphnid species: development of a preliminary chronic Zn *C. dubia* bioavailability model

4.1 Introduction

Chronic Zn toxicity to aquatic organisms has been shown to be dependent on the physico-chemistry of the surface water (e.g. Heijerick et al. 2002; 2005; De Schamphelaere et al. 2004; 2005). For daphnids, it has been shown that DOC, Ca, Mg, and Na all provide protection against chronic dissolved Zn toxicity (Heijerick et al. 2005; De Schamphelaere et al. 2005). To predict chronic Zn toxicity to *D.aphnia magna* under varying water chemistries a biotic ligand model (BLM) was developed (Heijerick et al. 2005). The chronic *D. magna* Zn BLM relates Zn toxicity to the activity of the free Zn^{2+} ion binding at the biotic ligand, which can be regarded as a cell-surface receptor for *D. magna*. Furthermore, the competition between Zn^{2+} and the cations H^{+}, Ca^{2+}, Mg^{2+}, and Na^{+} for binding at the Zn biotic ligand site is also taken into account (Heijerick et al. 2005). It is assumed that the biotic ligand stability constants, which express the binding affinity of a cation for the biotic ligand, can be extrapolated between (closely-)related species (Di Toro et al. 2001). In theory, the chronic Zn *D. magna* BLM should therefore also be applicable to predict chronic Zn toxicity to other invertebrates (Van Sprang et al. 2009). De Schamphelaere & Janssen (2010) previously showed that chronic Zn toxicity to the snail *Lymnaeae stagnalis* and the rotifer *Brachionus calyciflorus* can indeed be predicted with reasonable accuracy using the chronic Zn *D. magna* BLM. For *Ceriodaphnia dubia*, the cross-species applicability of the *D. magna* BLM has not yet been established. However, the latter is a prerequisite to be able to integrate the chronic Zn *D. magna* BLM in a metal mixture bioavailability model to predict chronic toxicity of Ni-Zn-Pb mixtures to *C. dubia* (Chapter 7). Therefore, we investigated the chronic toxicity of Zn to *C. dubia* in a series of Zn spiked natural waters differing in physico-chemistry. We then evaluated whether the chronic Zn *D. magna* BLM, adjusted for the sensitivity of *C. dubia*, can be used to accurately predict Zn toxicity in these field waters.

4.2 Materials and methods

4.2.1 Sampling of natural surface waters

Chronic Zn toxicity assays with *C. dubia* were conducted in natural waters. Natural water was collected from two locations in Belgium (l'Ourthe Orientale in Brisy and le Ruisseau de St. Martin in Bihain), three locations in the Netherlands (Ankeveense Plassen in Ankeveen, Beneden Regge in Ommen and Markermeer in Marken) and one location in France (Le Voyon in Trélon). Water was sampled on site using a 0.2 µm filter and collected in acid-washed poly-ethylene vessels. Upon arrival in the lab, the vessels were stored in total darkness at 4°C until further use. Zinc toxicity to *C. dubia* was investigated in these 6 natural waters. To investigate the individual effect of Ca and pH on Zn toxicity, two additional test waters (modified Brisy water) were included in the test design. Ca in one of the waters was increased by adding 0.75 mM Ca to the Brisy water, hereafter called the Brisy 1 mM Ca treatment. In the other water, pH of the Brisy water was increased to pH 7.8 using 0.6 mM $NaHCO_3$. Measured water chemistry in all natural waters used for toxicity testing is reported in Table 4.1.

Concentration series in each natural water contained a control (no Zn added) and 6 Zn concentrations, which were prepared by adding $ZnCl_2$. For the Brisy, Ankeveen, Regge, and Voyon natural waters nominal Zn concentrations ranged between 20 µg/L and 350 µg/L. For the Markermeer and Bihain natural waters nominal Zn concentrations ranged between 20 µg/L and 600 µg/L. All chemicals were purchased from VWR international.

Table 4.1. Main physicochemical characteristics of the test media used for chronic Zn toxicity testing with *Ceriodaphnia dubia*[a].

Test water ID	Name	pH	DOC (mg/L)	Ca (mg/L)	Mg (mg/L)	Na (mg/L)	K (mg/L)	Cl[b] (mg/L)	SO_4[b] (mg/L)	IC (mg/L)	Zn[c] (µg/L)
Bihain	Le Ruisseau de St. Martin (B)	6.4±0.1	12.4±1.6	3.7±0.0	1.4±0.0	5.7±0.2	1.1±0.0	24	6.5	1.1±0.3	20±2
Ankeveen	Ankeveense Plassen (NL)	8.0±0.1	11.4±2.6	44±0	12±0	62±0	8.2±0.0	115	60	17±0.8	10±4
Regge	Beneden Regge (NL)	8.3±0.1	16.4±1.9	71±0	8.7±0.1	44±0	12±0	62	55	32±0.8	15±3
Markermeer	Markermeer (NL)	8.1±0.0	9.5±1.3	59±1	19±0	96±1	12±0	72	84	23±0.4	7±3
Voyon	Le Voyon (F)	8.0±0.1	13.1±2.6	20±0	7.4±0.1	9.6±0.2	2.3±0.0	7.2	14	13±0.3	11±4
Brisy	L'Ourthe Oriental (B)	7.2±0.1	4.9±1.2	11±0	4.5±0.0	7.8±0	2.3±0.0	10	7.8	4.2±0.3	9±1
Brisy 1mM Ca	L'Ourthe Oriental (B)	7.2±0.2	4.8±1.1	34±0	4.5±0.0	8.0±0.1	2.4±0.0	56	7.7	4.3±0.3	9±1
Brisy pH7.8	L'Ourthe Oriental (B)	7.8±0.1	4.7±1.0	11±0	4.5±0.0	32±0	2.4±0.0	11	8.6	14±0.2	7±2

[a] Mean measured concentrations ± standard deviation are reported
[b] Cl and SO_4 was measured on a mixed sample of all test concentration for each individual water, therefore no standard deviation is available
[c] Measured backgorund dissolved Zn concentrations in the control treatments.
DOC= Dissolved organic carbon; IC= Inorganic carbon; B=Belgium; NL= the Netherlands; F=France

4.2.2 Ecotoxicity testing with *C. dubia*

Reproductive toxicity tests were conducted following the protocol of the United Sates Environmental Protection Agency (USEPA 2002a). Juveniles originated from an in-house isoclonal lab culture, which is maintained for more than 20 years at 25°C in activated carbon-filtered Ghent to which vitamins (75 µg/L thiamine, 1µg/L cyanocobalamin, and 0.75 µg/L biotin) and selenium (1 µg Se/L) are added. Daphnids were individually acclimated to the natural waters for one generation (1 week) in poly-ethylene cups containing 20 mL of the respective control natural waters (no Zn added and 1 daphnid per cup). Media were refreshed three times during the acclimation period. During acclimation and testing periods, daphnids were kept at 25°C under a 16h:8h light:dark cycle and daily fed with *Pseudokirchneriella subcapitata* ($2x10^5$ cells/mL) and a Yeast-Urtica-Trout Chow mixture (12 mg solids/L). Tests were initiated with juveniles originating from the third brood. Only juveniles originating from mothers that produced at least 20 juveniles during the first three broods and 8 juveniles in the third brood were used (USEPA 2002a). Tests were conducted in polyethylene cups containing 20 mL of the test medium. Juveniles (<24h old, 1 per replicate) were distributed among treatments as described in the USEPA protocol (2002a). Test media were renewed completely daily. Before renewal, fresh test media were adjusted to the required pH by adding dilute HCl or NaOH. Mortality and number of juveniles were scored daily. The toxicity tests were ended when at least 60% of the control animals had produced three broods.

4.2.3 Analytical chemistry

Samples of fresh medium (new medium immediately before transfer of the daphnid to the cup) for analysis of total and dissolved (0.45µm Acrodisc filter; PALL Life Science) Zn concentrations, organic carbon (OC) and inorganic carbon (IC) were taken on day 0 and day 6. Samples of old medium (sample taken of medium right after transfer of daphnid to new cup) for analysis of dissolved (0.45µm Acrodisc filter; PALL Life Science) Zn concentrations, OC and IC were taken on day 1 and day 7. Samples for metal analysis were acidified to 0.14 mol/L HNO_3. Zinc, Ca, Mg, Na and K concentrations were measured using inductive coupled plasma-optical emission spectroscopy (ICP-OES, iCAP 7000 series, Thermo Scientific). OC and IC were measured with a Total Organic Carbon analyser (TOC-5000, Shimadzu). Samples for analysis of Cl and SO_4 concentrations were taken from fresh medium on day 0 and measured using spectrophotometry

(Aquamate, Thermo Electron Corporation; Chloride: Merck, Spectroquant 1.14897.001; Sulphate: Merck, Spectroquant 1.14548.001). The pH of fresh and old media were measured daily with a pH glass electrode (Hanna Instruments).

4.2.4 Concentration response analysis

Concentration response analysis was conducted using the relative reproduction (total reproduction, i.e. number of juveniles per female, relative to the mean control reproduction) as endpoint. Median, 20% and 10% effective concentrations, expressed in µg dissolved Zn/L ($EC50_{Zndiss}$, $EC20_{Zndiss}$ and $EC10_{Zndiss}$, respectively), were calculated based on the average measured dissolved Zn concentration in fresh and old medium. Concentration response curves were fitted using the log-logistic concentration-response model with 2 parameters (Equation 4.1) in Statistica 3 (StatSoft).

$$\boldsymbol{y_i} = \frac{\mathbf{100}}{\mathbf{1}+\left(\frac{c_{\boldsymbol{Zndiss}}}{\boldsymbol{EC50_{Zndiss_i}}}\right)^{\boldsymbol{\beta Zn_i}}} \qquad (4.1)$$

In Equation 4.1, y_i is the predicted relative reproduction in test water *i*. c_{Zndiss} is the dissolved Zn concentration (µg/L). $EC50_{Zndiss,i}$ is the median effective concentration (µg dissolved Zn/L) in test water *i*. $\beta_{Zn,i}$ is the slope parameter of the concentration response curve of Zn in test water *i*.

4.2.5 Chemical speciation calculation

Chemical speciation of Zn^{2+}, Mg^{2+}, Ca^{2+} and Na^{+} was calculated using the Windermere Humic Aqueous Model VII (WHAM VII). WHAM VII uses the Humic Ion-Binding Model VII (Tipping et al. 2011) to model the competitive interactive between cations and dissolved organic matter (DOM). WHAM VII instead of the WHAM V, in which the *D. magna* Zn BLM was originally developed (Heijerick et al. 2005), was used to calculate chemical speciation in the toxicity tests, since it is the speciation software version which includes the most recent version of Humic Ion-Binding Model VII and can therefore be considered as the most state-of-the art version of WHAM. Furthermore, the chronic Zn *D. magna* BLM was shown to predict chronic Zn toxicity to *D. magna* with reasonable accuracy when using WHAM VII as speciation model (Chapter 3).

For speciation calculations, it was assumed that DOM contains 50% carbon on a weight basis. Furthermore, it was assumed that 65% of the DOM consists of reactive fulvic acid (FA), a fraction which is active in metal binding, and the other fraction being inert. As a consequence the measured dissolved organic carbon (DOC) concentration was multiplied by 1.3 to obtain the FA concentration needed for the input of the speciation calculations. Previous research has shown that assumptions of 60 to 70% active FA typically work best for predicting metal mixture toxicity (Tipping 2002). Additionally, the competing effects of Fe^{3+} for DOC binding sites have been considered by assuming that Fe^{3+} activity is controlled by $Fe(OH)_3$ using the default equation and solubility product embedded in WHAM VII. Finally, the default stability constants for complexation of Zn to inorganic ligands in WHAM VII were modified to those reported by the National Institute for Standards and Technology (Smith et al. 2004). These same assumptions, resulted also in reasonable predictions of chronic Zn toxicity to *D. magna* in WHAM VII (Chapter 3).

4.2.6 Bioavailability modelling

Chronic 7d-Zn toxicity to *C. dubia* was predicted using the chronic *D. magna* Zn BLM (Heijerick et al. 2005; Equation 4.2).

$$EC50_{Zn^{2+},i} = EC50^{*}_{Zn^{2+}}\left(1 + K_{CaBL}\{Ca^{2+}\}_i + K_{MgBL}\{Mg^{2+}\}_i + K_{NaBL}\{Na^{+}\}_i + K_{HBL}\{H^{+}\}_i\right) \quad (4.2)$$

In Equation 4.2, $EC50_{Zn2+,i}$ is the predicted 50% effective concentration of Zn^{2+} in test solution *i*. $EC50^{*}_{Zn2+}$ is the intrinsic sensitivities of the Zn BLM, which can be regarded as the $EC50_{Zn2+}$ of daphnid in a solution were all cationic competition effects are absent (Heijerick et al. 2005). K_{CaBL}, K_{MgBL}, K_{NaBL} and K_{HBL} are the stability constants for binding of Ca^{2+}, Mg^{2+}, Na^{+} and H^{+} to the Zn biotic ligand, respectively (Table 4.2). $\{Mg^{2+}\}$, $\{Ca^{2+}\}_i$, $\{Na^{+}\}$, and $\{H^{+}\}_i$ are the chemical activities of Mg^{2+}, Ca^{2+}, Na^{+} and H^{+} in test solution *i* (mol/L).

Since the chronic Zn sensitivity is different between *C. dubia* and *D.* magna, the intrinsic sensitivity was recalibrated specifically on the *C. dubia* toxicity data. The intrinsic sensitivity $EC50^{*}_{Zn2+}$ was calculated from the observed EC50 using Equation 4.3:

$$EC50^{*}_{Zn^{2+}} = \prod_{i}^{n}\left(\frac{EC50_{Zn^{2+},i,observed}}{1+K_{CaBL}\{Ca^{2+}\}_i+K_{MgBL}\{Mg^{2+}\}_i+K_{NaBL}\{Na^{+}\}_i+K_{HBL}\{H^{+}\}_i}\right)^{\frac{1}{n}} \quad (4.3)$$

In this equation, *n* is the number of test solutions considered. $EC50_{Zn2+,i,observed}$ is the observed Zn^{2+} activity in test solution *i* (mol/L). The intrinsic sensitivity was calibrated on the Zn toxicity data for *C. dubia* in all waters with pH<8, since the *D. magna* BLM was shown to be only valid up to pH 8 (De Schamphelaere et al. 2005). Toxicity data of the Bihain water were not considered in calculating the intrinsic sensitivities, since validity criteria of the *C. dubia* reproductive toxicity assay were not met in this water (see further). In the predictions of the $EC20_{Zndiss}$ and $EC10_{Zndiss}$ the Ankeveen water was not taken into account, since these values could not be reliably estimated.

Table 4.2. Biotic ligand stability constant parameters of the chronic Zn BLM for *Daphnia magna*[a] and the preliminary Zn *Ceriodaphnia dubia* bioavailability model[b]

	D. magna BLM[a]	*C. dubia* bioavailability model[b]
Log K_{CaBL}	3.2	3.2
Log K_{MgBL}	2.7	2.7
Log K_{NaBL}	1.9	1.9
Log K_{HBL}	5.8	-
S_{pH}	-	0.737

[a] Heijerick et al. 2005
[b] Present study
BLM=Biotic Ligand Model

The predicted 7d-$EC50_{Zn2+}$ were translated to 7d-$EC50_{Zndiss}$ with WHAM VII and compared to the observed 7d-$EC50_{Zndiss}$. Equation 4.2 and 4.3 describe the procedure to predict $EC50_{Zndiss}$. However, the same procedure can also be used to predict 7d-$EC20_{Zndiss}$ and 7d-$EC10_{Zndiss}$, by calculating a specific intrinsic sensitivity for the $EC20_{Zndiss}$ and $EC10_{Zndiss}$, respectively.

In order to calculate the observed slope of the pH function for *C. dubia*, $EC50_{Zn2+}$ were corrected for the presence of Ca^{2+}, Mg^{2+} and Na^{+} using Equation 4.4. The pH slope (S_{pH}) was determined based on the regression equation of the linear relationship between the logarithm of competitive corrected $EC50_{Zn2+}$, represented by $pEC50_{Zn2+,comp\ corrected}$, and pH.

$$EC50_{Zn^{2+},comp\ corrected} = \frac{EC50_{Zn^{2+},i,observed}}{\left(1+K_{MgBL}\times\{Mg^{2+}\}_i+K_{CaBL}\times\{Ca^{2+}\}_i+K_{NaBL}\times\{Na^{+}\}_i\right)} \quad (4.4)$$

In Equation 4.4, $EC50_{Zn2+,comp\ corrected}$ is the $EC50_{Zn2+}$ of the test water *i* that was corrected for the presence of Ca^{2+}, Mg^{2+} and Na^{+}.

4.3 RESULTS

4.3.1 Zn toxicity to *C. dubia*

The average control reproduction was in all tested waters higher than the minimum required by the USEPA test protocol, i.e. on average 15 juveniles per female, except in the Bihain water (Table 4.3). The concentration response data in the natural waters and the fitted log-logistic concentration response curves are shown in Figure 4.1. Zinc toxicity, expressed as $EC50_{Zndiss}$, ranged between 43 and 185 µg dissolved Zn/L (Table 4.3). Dissolved Zn toxicity was highest (i.e. lowest $EC50_{Zndiss}$) in the Brisy pH 7.8 water and lowest (i.e. highest $EC50_{Zndiss}$) in the Brisy 1 mM Ca water (Table 4.3). No reliable log-logistic concentrations response curve could be fitted for the Ankeveen water, due to the steepness of the concentration response. The $EC50_{Zndiss}$ for this water was derived from the regression between the observed relative response (%) at the 2 concentrations encompassing the EC50 and the log dissolved concentration. For the Brisy water, dissolved Zn toxicity decreased when Ca concentrations increased and Zn toxicity increased with increasing pH (Figure 4.1.B; Table 4.3).

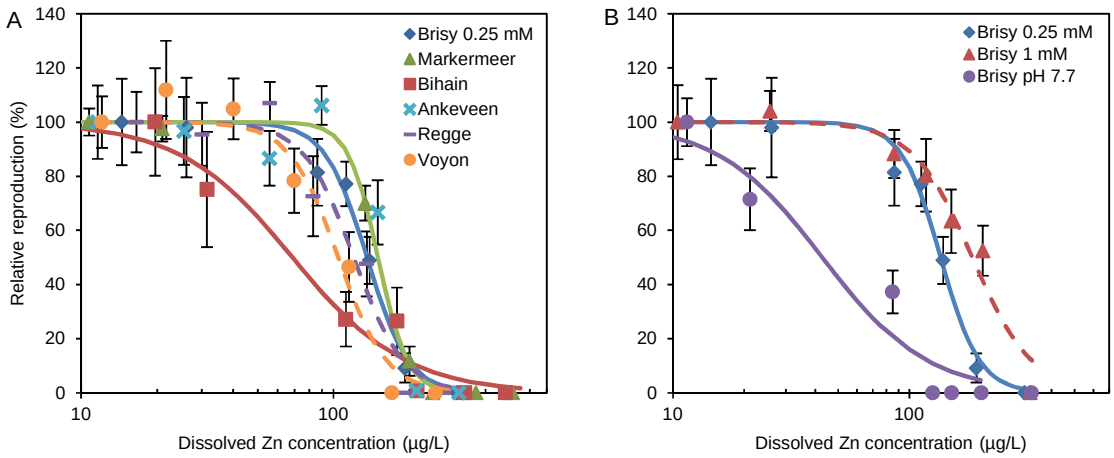

Figure 4.1. Concentration-response curves of dissolved 7d-Zn toxicity to *Ceriodaphnia dubia* in the unmodified natural waters (A) and the Brisy waters (B). Data points are the mean reproduction relative to the control reproduction. Plotted error bars represent standard errors. Fitted curves are log-logistic concentration response curves (Equation 4.1). No reliable log-logistic concentration response curve could be fitted for the Ankeveen water.

Table 4.3. Control reproduction and parameters (50%, 20%, and 10% effective concentrations, and slopes) of the concentration response curve for chronic (7d) reproductive toxicity of Zn to *Ceriodaphnia dubia*?

Test water ID	Control reproduction[a]	$EC10_{Zndiss}$[b] (µg/L)	$EC20_{Zndiss}$[b] (µg/L)	$EC50_{Zndiss}$[b] (µg/L)	β_{Zndiss}[c]
Markermeer	24.7±1.2	111 (102-122)	124 (116-132)	150 (143-158)	7.33±1.07
Bihain	13.6±2.7	22 (9-52)	34 (17-65)	69 (44-106)	1.94±0.52
Ankeveen	24.6±3.3	-	-	164[d] (150-213)	-
Regge	25.6±2.9	73 (52-103)	88 (68-113)	120 (103-140)	4.40±1.23
Voyon	18.5±1.8	64 (43-97)	77 (57-104)	104 (88-124)	4.53±1.57
Brisy	16.3±2.6	89 (69-115)	104 (87-125)	136 (121-152)	5.24±1.56
Brisy 1mMCa	21.9±3.0	98 (70-137)	124 (98-156)	185 (158-216)	3.44±0.90
Brisy pH 7.8	22.8±2.0	14 (9-22)	21 (15-31)	43 (33-56)	1.96±0.29

[a] Average number of juveniles per female ± standard error
[b] For EC10, EC20 and EC50 95% confidence intervals are reported between brackets.
[c] β±standard error is reported
[d] For the Ankeveen water, no reliable EC10, EC20 and EC50 could be calculated with the log-logistic concentration response due to the steepness of the concentration response. The EC50 was derived from the regression between the observed relative response (%) at the 2 concentrations encompassing the 50% effect level and the log dissolved concentration. The reported confidence limit for this EC50 are the 2 concentrations that encompass the EC50
EC10=10% effective concentration; EC20=20% effective concentration; EC50=median effective concentration; β=slope of the log-logistic concentration response curve

4.3.2 Prediction of Zn toxicity to *C. dubia* using the *D. magna* BLM

Intrinsic sensitivities of the *D. magna* BLM were 0.285, 0.151, and 0.115 µmol/L for the EC50, EC20, and EC10, respectively. Zinc toxicity predicted with the *D. magna* BLM is plotted as a function of the observed Zn toxicity in Figure 4.2. Although the average prediction error on $EC50_{Zndiss}$ was lower than 2-fold when only the waters with pH≤8 were considered, median effective concentrations of only three out of five waters were predicted within 2-fold error (Table 4.4). For the $EC20_{Zndiss}$ and $EC10_{Zndiss}$, Zn toxicity in only one out of four waters was predicted within 2-fold error. Similar prediction capacities were observed when the waters with pH>8 were also considered (Table 4.4).

Figure 4.3 shows the observed and predicted $EC50_{Zndiss}$ in the Brisy waters as a function of pH and Ca. Although the observed Zn toxicity increases with increasing pH, the *D. magna* BLM predicts a slightly decreasing toxicity when pH increases. The *D. magna* BLM predicts the observed trend of decreasing toxicity with increasing Ca concentration, but the Zn toxicity is overestimated with 1.7- to 2.8-fold error.

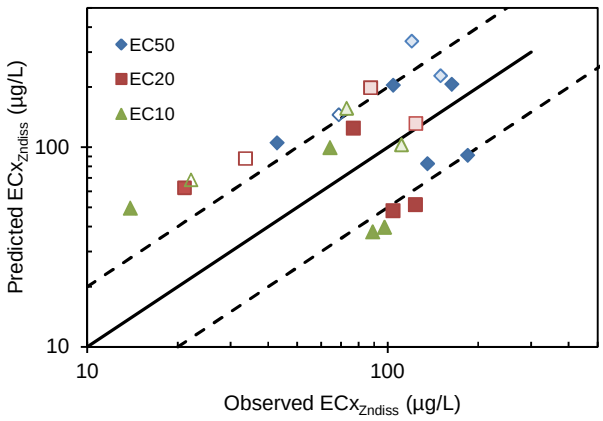

Figure 4.2. Predicted versus observed effect concentrations resulting in x% reduction of reproduction (7d-ECx; expressed as µg dissolved Zn/L) for *Ceriodaphnia dubia* in natural waters. Predictions were made using the *Daphnia magna* Zn BLM (Eq. 4.2) linked to WHAM VII. Dashed line represents a difference of a factor 2 between the observed and predicted data. The full line represents a perfect fit between observed and predicted data. Open data points represent the ECx_{Zndiss} of the Bihain water, which was not taken into account for intrinsic sensitivity calibration. Lighter colored symbols are the ECx_{Zndiss} of the waters with pH>8 (also not taken into account for model calibration).

Table 4.4. Prediction capacity of the chronic Zn *Daphnia magna* BLM in predicting dissolved Zn reproductive toxicity (7d) to *Ceriodaphnia dubia*

	Waters with pH≤8			All waters except Bihain		
	EC50[a]	EC20[b]	EC10[b]	EC50[a]	EC20[b]	EC10[b]
Number of waters	5	4	4	7	6	6
Mean prediction error	1.87	2.29	2.48	1.96	2.08	2.19
Median prediction error	1.96	2.28	2.41	1.96	2.21	2.26
Maximum prediction error	2.47	2.97	3.56	2.83	2.97	3.56
Predicted within 2-fold (%)	60	25	25	57	33	33

[a] The Bihain water was not taken into account in calculations of the prediction capacities for the EC50
[b] The Bihain and Ankeveen waters were not taken into account in calculations of the prediction capacities for the EC20 and EC10
EC10=10% effective concentration; EC20=20% effective concentration; EC50=50% effective concentration

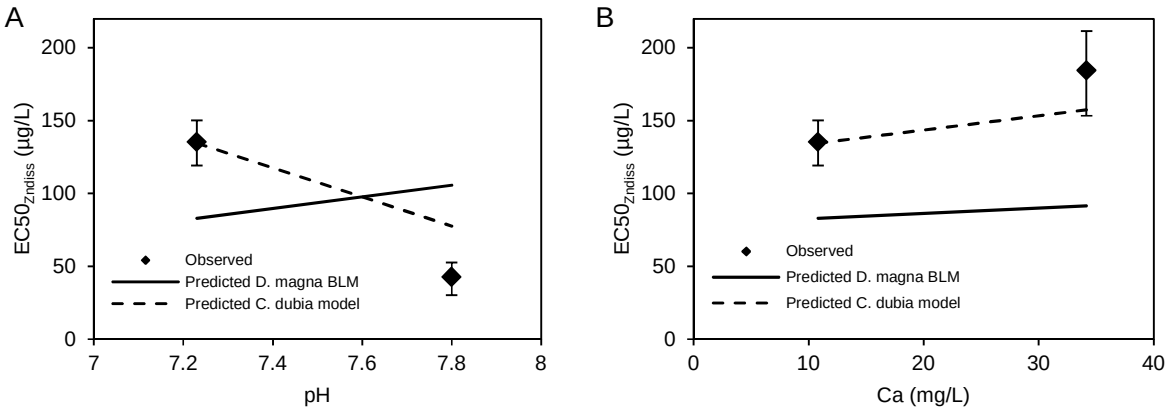

Figure 4.3. Observed (symbols) and predicted (lines) 7d-$EC50_{Zndiss}$ for reproductive toxicity of *Ceriodaphnia dubia* in the Brisy test waters as a function of pH (A) and Ca (B). Plotted error bars denote 95% confidence intervals on observed $EC50_{Zndiss}$. Full lines represent predictions of the chronic Zn *Daphnia magna* BLM (Equation 4.2; Heijerick et al. 2005). Dashed lines represent predictions of the preliminary Zn *C. dubia* bioavailability model (Equation 4.5; present study).

4.4 Discussion

4.4.1 Zn toxicity to *C. dubia*

The USEPA protocol requires that the average reproduction in the controls should be at least 15 juveniles per female (USEPA 2002a). In all natural waters this validity criterion was met, except in the Bihain water. The Bihain water originates from a small stream that drains a highland peat swamp. This water combines a low pH with very low concentrations of cations. Therefore, it is possible that the characteristics of this water are at the boundaries of the physiological conditions needed to sustain *C. dubia* reproduction. Additionally, background Zn concentrations in this water were rather high (Table 4.1) compared with the Zn toxicity (see further). As a consequence, the Bihain water was not considered in the evaluation of the prediction performance of the Zn *D. magna* BLM for *C. dubia.*

The observed 7d-$EC50_{Zndiss}$ ranged between 43 and 185 µg dissolved Zn/L. These values are in line with previously reported values for reproductive impairment of Zn to *C. dubia*. Belanger & Cherry (1990) observed a reproductive impairment of approximately 50% at 100 µg nominal Zn/L in a series of natural waters, but they did not calculate effective concentrations.

Based on the results of the present study, *C. dubia* seems to be more sensitive to Zn than *D. magna*. Toxicity to *D. magna,* expressed as $EC50_{Zndiss}$, in the same set of natural waters as used in the present study ranged between 112 and 536 µg dissolved Zn/L (De Schamphelaere et al. 2005). It has earlier been reported that *C. dubia* is among the most sensitive invertebrates for chronic Zn toxicity (Van Sprang et al. 2009).

Based on the toxicity tests in the Brisy water, a few general conclusions about the influence of pH and Ca on chronic Zn toxicity can be drawn. When the pH increased from 7.2 to 7.8 , the observed toxicity, expressed as $EC50_{Zndiss}$ increased with more than 3-fold. A similar trend of increasing chronic dissolved Zn toxicity with increasing pH was previously observed for the fish *Oncorhynchus mykiss* (De Schamphelaere & Janssen 2004b) and the algae *P. subcapitata* (Heijerick et al. 2002), but was not observed for *D. magna* (Heijerick et al. 2005). For the rotifer *Brachionus calyciflorus* and the snail *Lymnaea stagnalis* chronic Zn toxicity even decreased with increasing pH (De Schamphelaere & Janssen 2010). The effect of pH on chronic Zn toxicity may be attributed to two different mechanisms which counteract each other. First, pH affects the speciation of Zn, resulting in an increase in the bioavailable and thus toxic Zn^{2+} ions when pH decreases. Alternatively, low pH also promotes the competition between H^{+} and Zn^{2+} at the Zn biotic ligand sites, and as such protects against Zn^{2+} toxicity (Heijerick et al. 2005).

A modest increase in Ca from 11 mg/L to 35 mg/L, resulted in a 1.4-fold decrease in Zn toxicity, expressed as $EC50_{Zndiss}$. This is in agreement with the general observed protective effect of Ca on chronic Zn toxicity to *D. magna*, *O. mykiss* and *P. subcapitata* (Heijerick et al. 2002; 2005; De Schamphelaere et al. 2004). Indeed, it has been argued that hypocalcaemia, through the disruption of the Ca homeostasis is a likely mechanism of Zn toxicity to *D. magna* (Muyssen et al. 2006). In fish, (acute) Zn toxicity has been linked with the competitive inhibition of the Ca^{2+} uptake at the apical membrane of the gill epithelia (Hogstrand et al. 1995). However, it has been acknowledged that Zn may possibly interfere with the Ca homeostasis at several additional levels (Hogstrand 2012).

4.4.2 Extrapolation of the *D. magna* BLM for *C. dubia*

Although the intrinsic sensitivities of the Zn *D. magna* BLM were specifically calibrated on the toxicity data of *C. dubia*, prediction errors were relatively large (Table 4.4). Only three out of five $EC50_{Zndiss}$ were predicted within two-fold error, when the waters with pH≤8 were considered. The performance of the model in predicting 20% and 10% effective concentrations was even worse: only one out of four $EC20_{Zndiss}$ and $EC10_{Zndiss}$ were predicted within two-fold error. The average prediction errors of the *D. magna* BLM for the invertebrates *L. stagnalis* and *B. calyciflorus* were considerably smaller (De Schamphelaere & Janssen 2010), although these species even belong to a different phylum. Figure 4.3.A gives a possible explanation for the failed extrapolation of the *D. magna* BLM to *C. dubia*. The *D. magna* BLM predicts a decrease in toxicity with increasing pH, while chronic Zn toxicity actually increases with increasing pH. The latter suggests that the effect of pH on Zn toxicity is different between *D. magna* and *C. dubia.*

4.4.3 Development of a preliminary Zn *C. dubia* bioavailability model

Figure 4.4 shows the log $EC50_{Zn2+}$ corrected for the competitive effects of Ca^{2+}, Mg^{2+} and Na^{+} (using Eq. 4.4) as a function of pH. The slope of the pH function for *C. dubia* between pH 7.2 and pH 8.3 is 0.737. Using this pH slope a species-specific Zn bioavailability model for *C. dubia* can be developed. In this preliminary bioavailability model, the effect of pH on Zn^{2+} toxicity will be modelled as a log-linear pH effect superimposed on the competitive effects of Ca^{2+}, Mg^{2+} and Na^{+} (Equation 4.5). To this model will hereafter be referred as the preliminary Zn *C. dubia* bioavailability model.

$$EC50_{Zn^{2+},i} = 10^{-(Q50_{Zn^{2+}} + S_{pH,C.dubia} pH_i)} \left(1 + K_{CaBL}\{Ca^{2+}\}_i + K_{MgBL}\{Mg^{2+}\}_i + K_{NaBL}\{Na^{+}\}_i\right) \quad (4.5)$$

In Equation 4.5, $Q50_{Zn2+}$ is the intrinsic sensitivity of the preliminary *C. dubia* bioavailability model. This intrinsic sensitivity can be regarded as the intercept of the linear relationship between the negative logarithm of the $EC50_{Zn2+}$, after correction for Ca^{2+}, Mg^{2+} and Na^{+} competition, and pH. $S_{pH,C.dubia}$ is he slope of the latter relationship. Finally, pH_i is the pH of solution *i.* In this model, it is assumed that the competitive interactions between Zn^{2+} and the cations Mg^{2+}, Ca^{2+} and Na^{+} are the same for *D. magna* and *C. dubia*. It is currently not known if this assumption holds true. However, it has been reported that the stability constants for binding

of Mg^{2+}, Ca^{2+} and Na^{+} to the Zn biotic ligand are relatively similar between fish and *D. magna*, suggesting that similar mechanisms drive Zn toxicity in fish and *D. magna* (Van Sprang et al. 2009). Thus, until the effects of Ca, Mg and Na on Zn toxicity to *C. dubia* are further clarified, this assumption seems reasonable. An overview of the model parameters of the preliminary Zn *C. dubia* bioavailability model is given in Table 4.2.

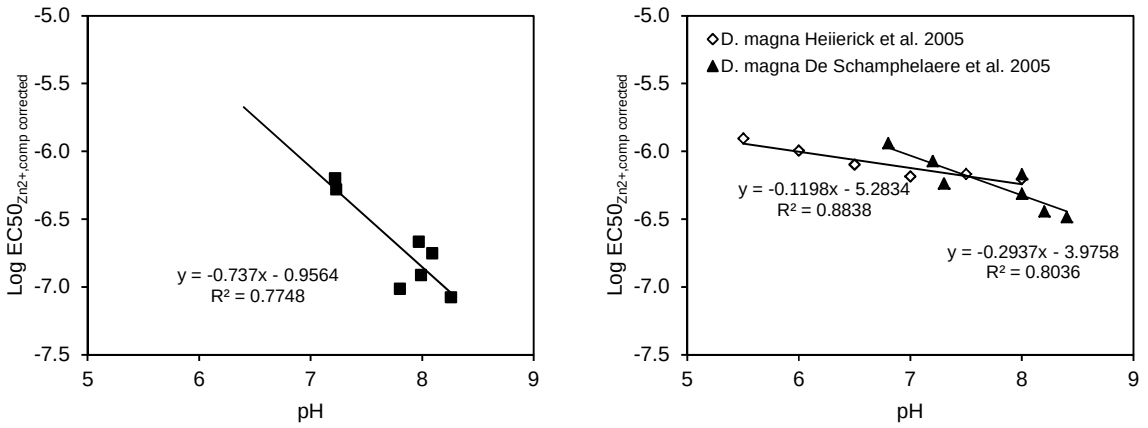

Figure 4.4. The effect of pH on WHAM VII calculated log 7d-$EC50_{Zn2+}$ of *Ceriodaphnia dubia* (present study; A) and on log 21d-$EC50_{Zn2+}$ of *Daphnia magna* (B; data from the pH test series in Heijerick et al. 2005 and natural waters series in De Schamphelaere et al. 2005; using the speciation assumptions as described in section *4.2.5 Chemical speciation calculation*) after correction for the competitive effects of Ca^{2+}, Mg^{2+} and Na^{+} (Equation 4.4), represented by log($EC50_{Zn2+,comp\ corrected}$).

The intrinsic sensitivities of the preliminary Zn *C. dubia* bioavailability model are reported in Table 4.5. Using this model, all $EC50_{Zndiss}$ were predicted within 2-fold error (Figure 4.5) and the median and mean prediction error were 1.2 and 1.3 (Table 4.5), respectively. $EC20_{Zndiss}$ and $EC10_{Zndiss}$ were slightly less well predicted, but the mean and median prediction errors were still reasonable. Toxicity in the Bihain water, which was not taken into account for intrinsic sensitivity calculations, was underestimated by at least 9-fold. This can possibly be the consequence of an increased Zn toxicity caused by physiological stress due to the low major ion concentrations in the natural water and/or low pH. Alternatively, it could also be that the pH function does not accurately reflect the effect of pH on Zn toxicity for pH below ~7. The Zn *C. dubia* bioavailability model predicted the effect of pH and Ca on Zn toxicity in the Brisy waters relatively accurate (Figure 4.3). The preliminary Zn *C. dubia* bioavailability has been shown to be valid in the pH

range from pH 7 to 8.3. However, application of the model to waters below pH 7 must be avoided.

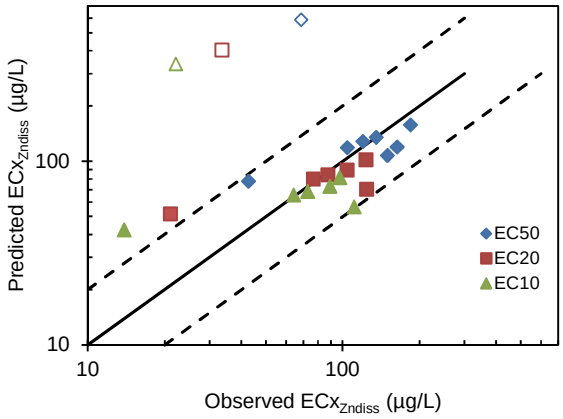

Figure 4.5. Predicted versus observed effect concentrations resulting in x% reduction of reproduction (7d-ECx; expressed as µg dissolved Zn/L) for *Ceriodaphnia dubia* in natural waters. Predictions were made using the preliminary *C. dubia* bioavailability model (Eq. 4.5) linked to WHAM VII. Dashed lines represents a difference of a factor 2 between the observed and predicted data. The full line represents a perfect fit between observed and predicted data. Open data points represent the ECx$_{Zndiss}$ of the Bihain water, which was not taken into account for intrinsic sensitivity calibration.

Table 4.5. Prediction performance of the preliminary chronic Zn *Ceriodaphnia dubia* bioavailability model in predicting dissolved Zn reproductive toxicity (7d) to *C. dubia*

	EC50[a]	EC20[b]	EC10[b]
n	7	6	6
Intrinsic sensitivity (Qx$_{Zn2+}$)	0.96	1.17	1.27
Mean prediction error	1.28	1.45	1.59
Median prediction error	1.17	1.19	1.21
Maximum prediction error	1.81	2.45	3.03
%predicted within 2-fold	100	83	83

[a] The Bihain water was not taken into account in calculations of the prediction capacities for the EC50
[b] The Bihain and Ankeveen waters were not taken into account in calculations of the prediction capacities for the EC20 and EC10
EC10=10% effective concentration; EC20=20% effective concentration; EC50=median effective concentration

The log-linear model structure of the preliminary Zn *C. dubia* bioavailability model (Eq. 4.5) deviates from the typical linear BLM-type equations, wherein the competitive effects of H^+ are modelled as a single-site biotic ligand competition, such as in the *D. magna* BLM for Zn (Heijerick et al. 2005; Equation 4.2). However, the log-linear model structure has been used, for instance, to model chronic Zn toxicity to algae (De Schamphelaere & Janssen 2005) and chronic

Ni toxicity to *D. magna* (Deleebeeck et al. 2008) and *C. dubia* (De Schamphelaere et al. 2006). Moreover, Heijerick and colleagues (2005) reported that the effect of pH on chronic Zn toxicity to *D. magna* is described more accurately by a log-linear relationship between pH and Zn^{2+} toxicity than by the linear relation between H^+ activity and Zn^{2+} toxicity, which was eventually used in the chronic Zn *D. magna* BLM.

The slope of the log-linear relation between Zn^{2+} toxicity and pH for *C. dubia* in the present study is only slightly steeper than the one of *P. subcapitata* ($S_{pH,P.subcapitata}$=0.652; Heijerick et al. 2002), but at least twice as steep as the slope of the effect of pH on Zn toxicity to *D. magna* (Figure 4.4.B). For Ni, a similar difference in the steepness of the effect of pH on Ni^{2+} toxicity between *D. magna* (S_{pH}=0.3335) and *C. dubia* (S_{pH}=0.86) was observed (De Schamphelaere et al. 2006). The difference in the effect of pH on Ni and Zn toxicity between these two relative closely related species deserves further attention, since current bioavailability normalization approaches in ecological risk assessment procedures for metals are based on the assumptions that the bioavailability models developed for one species can be used to predict toxicity for other related species (Van Sprang et al 2009; Schlekat et al. 2010). For instance, the chronic Zn *D. magna* BLM has been used to normalize toxicity data for all invertebrates (Van Sprang et al. 2009). As such, it is implicitly assumed that the competitive effects between Zn^{2+} and other cations, such as Mg^{2+}, Ca^{2+}, H^+ and Na^+ for binding at the biotic ligand are similar between invertebrates and that only metal sensitivities vary between invertebrate species. The present study suggests that this assumption might be too simple in reality. However, it is not realistic to develop a species-specific bioavailability model for all possible species for which metal toxicity data exist, since metal toxicity data sets are typically species-rich (e.g. in the chronic Zn toxicity database 19 species are included; Van Sprang et al. 2009). Therefore, the uncertainties associated with the choice between the *D. magna* or *C. dubia* model in the Zn bioavailability normalization approaches should be addressed. In the Ni risk assessment process, a step evaluating which of the two daphnid models is the most conservative for invertebrate species for which cross-species applicability of a bioavailability model has not been established has been built in (DEPA 2008).

In conclusion, the chronic Zn *D. magna* BLM underestimates the effect of pH on Zn toxicity. The preliminary chronic Zn *C. dubia* bioavailability model developed in the present study predicted Zn toxicity in a series of natural waters relatively accurately, i.e. all within 2-fold error. The

preliminary Zn *C. dubia* bioavailability has been shown to be valid in the pH range of 7 to 8.3. However, the prediction performance of the model in waters below pH 7 should be further investigated

5

Mixture toxicity of nickel and zinc to *Daphnia magna* is non-interactive at low effect sizes, but becomes synergistic at high effect sizes

Redrafted from:

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5. Mixture toxicity of nickel and zinc to *Daphnia magna* is non-interactive at low effect sizes, but becomes synergistic at high effect sizes

5.1 Introduction

Surface waters can be contaminated with mixtures of metals originating from anthropogenic activities. While the chronic effects of individual metals to aquatic organisms have already been extensively characterized (e.g. De Schamphelaere & Janssen 2004b; Heijerick et al. 2005; Grosell et al. 2006a; Deleebeeck et al. 2008), chronic effects of metal mixtures have not. Two general reference models are commonly used for the description of mixture toxicity: concentration addition (CA) and independent action (IA) (Jonker et al. 2005). The CA model assumes that substances have a similar mode of action and that a substance in a mixture can be exchanged for other substances without changing the overall mixture toxicity, as long as the sum of toxic units of the mixture does not change. The IA model assumes that the substances in a mixture have a dissimilar mode of action and the response of the mixture substances is calculated as a product of responses from each of the substances. Both models assume that there is no interaction among the substances in the mixture, i.e. "non-interaction". However, when chemical substances are interacting with each other, the toxicity of a mixture can be higher or lower than expected from that of the individual substances in the mixture based on the reference model, i.e. synergism or antagonism, respectively (Jonker et al. 2005). Both non-interaction, synergism and antagonism have been observed in toxicity assays with metal mixtures (Norwood et al. 2003; Vijver et al. 2011), and in some cases within the same metal combination both non-interactive and interactive effects were found (Sharma et al. 1999). Most published metal mixture studies are limited to acute effects (e.g., Ferrer et al. 2006; Hatano & Shoji 2008; Mebane et al. 2012; Zhu et al. 2011), while chronic effects have rarely been considered (but see Lock & Janssen 2002; Cooper et al. 2009; Norwood et al. 2013).

Although mixture toxicity has, to date, rarely been incorporated in risk assessment (Backhaus & Faust 2012), it is anticipated that it will be required to account for mixture toxicity in future regulatory risk assessments (CEU 2009). Uncertainties associated with the type of metal mixture interactions occurring and the lack of suitable chronic studies hinder the incorporation of metal mixture toxicity in risk assessment. Therefore, more knowledge on how metals interact during

chronic exposure to metal mixtures is needed. The objective of this study was to measure chronic metal mixture toxicity and to test whether metals act interactively or not. Because the reproducibility of interactive mixture effects has previously been debated (Cedergreen et al. 2007), we investigated the chronic toxicity and interactive effects of binary Ni-Zn mixtures during two independent large-scale experiments using the 21 day *Daphnia magna* reproduction test in a full-factorial design. Both experiments used a similar test medium, but with organic matter of different origin (Aldrich Humic Acid (AHA) and natural Dissolved Organic Carbon (DOC)). The type of DOC was varied between both experiments since DOC is one of the most important water chemistry variables determining metal toxicity. Furthermore, the protective abilities of DOC on metal toxicity varies greatly between the different types of DOC (Wood et al. 2011). Nickel & Zn were selected because other studies suggested interactions among these metals. Komjarova and Blust (2008) showed that the uptake of Ni by *D. magna* decreased at increasing concentrations of Zn, possibly through competitive inhibition at the level of uptake. A similar inhibitory effect of elevated Zn concentrations on Ni uptake was reported for the fish *Danio rerio* (Komjarova & Blust 2009), the bacteria *Bradyrhizobium japonica* (Fu & Maier 1991), the alga *Chlamydomonas reinhardtii* (Worms & Wilkinson 2007), and the higher plant *Sesbania drumondii* (Israr et al. 2011). Conversely, Ni uptake was enhanced in the presence of elevated concentrations of Zn for the freshwater mussel *Dreissena polymorpha* (Bourgeault et al. 2012). Additionally, the uptake of Zn by *Daphnia* (Komjarova & Blust 2009) and plants (Israr et al. 2011) was decreased in the presence of Ni. Therefore, we expected to observe primarily antagonistic interactions on toxicity in the Ni-Zn mixtures.

5.2 Methods

5.2.1 Test medium and test design

Two separate *D. magna* 21-day reproduction tests were conducted, both with a binary Ni-Zn mixture. The tests were conducted in modified M4 medium (Elendt & Bias 1990). The water hardness of the original M4 medium (370 mg $CaCO_3$/L) was reduced to 180 mg $CaCO_3$/L for both toxicity tests. Measured Ca and Mg concentrations in the exposure medium were 1.4 and 0.4 mM, respectively. The Na_2-EDTA in the original medium was replaced in one test with either 0.8 mg/L DOC originating from Aldrich Humic Acid (Sigma-Aldrich, St-Louis, MO, USA), hereafter

called the 'AHA test', or 4 mg/L natural dissolved organic carbon (DOC), hereafter called the 'natural DOC test', since the addition of DOC results in an environmentally more realistic medium than when EDTA is used (Bossuyt & Janssen 2003). To collect natural DOC, natural water was sampled from the Schwarzbach (Küchelsheid, Belgium) (a small river containing low Ca (1.8 mg/L) and moderate DOC (8.9 mg/L) concentrations) and transported to the laboratory. The dissolved organic matter (DOM) was concentrated in the laboratory using a reverse osmosis system as described by De Schamphelaere et al. (2003) and stored in a polyethylene barrel at 4°C in total darkness until further use. Samples of this stock solution were taken and used for analysis of DOC, major cation and trace metal concentrations (see Section 5.2.3). Trace metal concentrations in the test medium resulting from the DOC stock solution were low, except for Zn. Therefore, we omitted the addition of Zn in the original M4-medium for the natural DOC test, since the natural DOC source provided already an appropriate background Zn concentration in the medium to sustain *D. magna* reproduction. In both experiments a background concentration of approximately 20 µg dissolved Zn/L was present in the control, as it was previously shown that media with very low background zinc concentration can result in Zn deficiencies and a decreased daphnid fitness (Muyssen & Janssen 2001). Background Ni concentrations in both mediums were below the detection limit (DL=0.5 µg Ni/L). Finally, for the natural DOC test, we also omitted the stock C solution of the M4-medium, containing Na_2SiO_3, $NaNO_3$, KH_2PO_4 and K_2HPO_4, because these substances enhance algal growth and are not needed for daphnid growth or reproduction. The omission of these nutrients did not influence Ni^{2+} and Zn^{2+} speciation (data not shown). The chemical composition of the test media is given in Appendix B (Table B.1).

The modified M4-medium was aerated for 2 days prior to spiking. After spiking solutions were left to equilibrate for 1 day at 20°C. The interactive effects of Ni and Zn in the AHA test were tested in a full-factorial design with 6 Zn (range 20-560 µg nominal Zn/L) and 7 Ni (range 0-180 µg nominal Ni/L) concentrations. For the natural DOC test a full-factorial design with 7 Zn (range 20-560 µg nominal Zn/L) and 7 Ni (range 0-320 µg nominal Ni/L) concentrations was used. In total 42 (6x7-design) and 49 (7x7-design) treatments of these two metals were tested in the AHA- and natural DOC-test, respectively (Figure 5.1). The treatment concentrations were prepared by adding $ZnCl_2$ and $NiCl_2$ (analytical grade). All chemicals were purchased from VWR International (Leuven, Belgium).

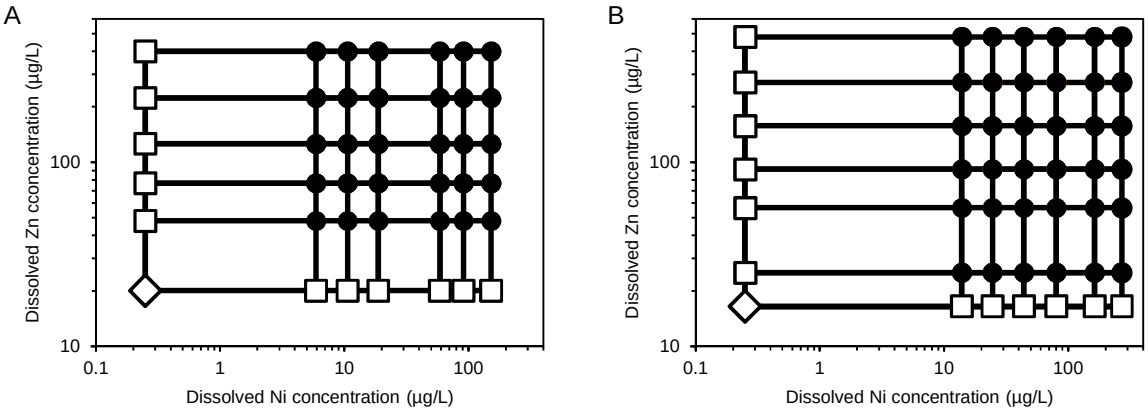

Figure 5.1. Test design for the AHA- (A; 6 Zn x 7 Ni-concentrations) and for the natural DOC- (B; 7 Zn x 7 Ni-concentrations) test series used to investigate the toxicity of binary Ni-Zn mixtures on the reproduction of *Daphnia magna*. Concentrations are the average of measured dissolved concentrations in new and old media. Filled symbols indicate mixture treatments of Ni and Zn. Open squares indicate individual Ni or Zn toxicity treatments also acting as so-called mixture controls. The diamond indicates the absolute control treatment (no Ni added, Zn at its background concentration, i.e. ±20 µg/L).

5.2.2 Ecotoxicity testing

The interactive effects between Ni and Zn were tested using the 21 day chronic reproduction assay as described in OECD protocol No 211 (OECD 2012). *D. magna* juveniles originated from an in-house isoclonal laboratory culture (clone K6) which has been maintained for more than 20 years at 20°C in activated carbon filtered Ghent city tap water to which 1 µg Se/L, 75 µg/L thiamine, 1 µg/L cyanocobalamine and 0.75 µg/L biotine are added. Daphnids were acclimated in aquaria containing 5L of the control test media (no Ni and background concentration of Zn) for one generation prior to test initiation. Media were renewed three times a week. During acclimation and ecotoxicity testing, daphnids were kept at 20°C, under a light/dark-cycle of 16h/8h and fed with a mixture of the algae *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3:1 ratio (on a cell number basis; 'algae mix').

All combinations within a test were run simultaneously to exclude any possible interference with subsequent data interpretation due to temporal sensitivity variation (Delaender et al. 2009). Tests were initiated with third brood juveniles which were less than 24h old (±15 days after initiation of the acclimation). Tests were conducted in polyethylene cups containing 50 mL test medium. Every treatment consisted of 10 replicates, except the control treatment in the natural DOC test

(see further). Treatments were distributed over 7 and 8 test plates for the AHA and natural DOC test, respectively. In the natural DOC test, every test plate received also one row of 10 control replicates (no Ni and background concentration of Zn), to account for possible plate differences in control reproduction. Hence, in total 420 (10 replicates of each of the 41 metal treatments plus 10 controls) experimental vials were used in the AHA test and 560 (10 replicates of each of the 48 metal treatments plus 80 controls) in the natural DOC test, respectively. During the ecotoxicity test, daphnids were fed daily with 250, 500 and 750 µg dry weight algae mix per exposure vial (50 mL) in the first, second and third week, respectively. Test media were renewed three times a week. Before renewal, fresh test media were adjusted to the required pH (7.6, range 7.5-7.7) by adding dilute HCl or NaOH. Mortality and number of juveniles were scored daily.

5.2.3 Analytical chemistry

During the exposure period, samples of fresh (sample of new medium just before transfer of daphnids to the cup) and old (sample taken of medium just after transfer of daphnids to a new cup) test media of all treatments were collected weekly for analysis of total (only of fresh medium) and filtered concentrations of Ni and Zn. Total and filtered (0.45 µM, Acrodisc, PALL Life Sciences, Port Washington, NY, USA) samples of fresh media were taken on day 0, 6 and 13. Filtered samples of old media were taken on day 2, 8 and 15. Weekly samples for total (only for fresh medium) and dissolved (in)organic carbon analysis were taken for 14 random chosen combinations in the natural DOC-test. Samples for Ni and Zn measurements were acidified to 0.14 mol/L HNO_3 (Normatom quality, VWR Prolabo, Leuven, Belgium). Nickel concentrations ≤ 100 µg/L were measured using graphite furnace atomic absorption spectrophotometry (GFAAS Furnace Autosampler, Thermo Fisher Scientific Inc., Waltham, MA, USA; Reference Material TM-24.3, lot 0510 (Environment Canada): Limit of Quantification 1 µg Ni/L; Method Detection Limit 0.5 µg Ni/L). All Zn and Ni concentrations >100 µg/L were measured using flame atomic absorption spectrophotometry (SpectrAA100, Varian, Mulgrave, Australia; Reference Material TMDA-70, lot 0310 (Environment Canada): Limit of Quantification 60 µg Ni/L and 20 µg Zn/L; Method Detection Limit 20 µg Ni/L and 6 µg Zn/L). DOC (Dissolved Organic Carbon) and DIC (Disolved Inorganic Carbon) were measured with a Total Organic Carbon analyzer (TOC-5000, Shimadzu, Duisburg, Germany; Limit of Quantification 1.5 mg DOC/L;

Method Detection Limit 0.5 mg DOC/L). For a random subset of samples trace metal and major ion concentrations were measured with ICP-OES (Perklin Elmer 3300 DV). The pH of fresh and old media was measured on renewal days with a pH glass electrode (P407, Consort, Turnhout, Belgium).

5.2.4 Speciation calculations

Speciation of Ni, Zn and other ions was calculated with the software package WHAM (Windermere Humic Aqueuos Model) VI (Tipping 1998), based on the average of measured dissolved Ni and Zn concentrations in fresh and old medium per treatment and the water chemistry reported in Table B.1. The default stability constants of Ni, Cu and Zn for inorganic carbonate-complexes were adapted to those of National Institute of Standards and Technology (Table 3.1). Furthermore, the binding assumptions of both Ni and Zn with fulvic acid (FA) were updated to the values reported in Van Laer et al. (2006) for Ni and NiOH ($DLK_{2(FA)}$=2.35) and Cheng et al. (2005) for Zn and ZnOH ($LogK_{MA(FA)}$=1.8). For the speciation calculations we assumed that DOM contained 50% carbon on a weight basis (Ritchie & Perdue 2003). Furthermore, for the natural DOC we assumed that 65% of the DOM is reactive and behaves as isolated FA. Tipping (2002) showed that for predicting metal toxicity in natural waters, assumptions between 60% and 70% of active FA work best. Consequently, to obtain the FA concentration (mg/L) for the speciation calculation input the measured DOC concentration (mg C/L) multiplied by a factor 1.3 was used. In the AHA test, DOC concentrations of the medium were not measured. Therefore, nominal DOC concentrations were used for the speciation calculations in the AHA test series. For the Aldrich HA (AHA), we assumed that this HA corresponds to 100% reactive HA. Hence, for the AHA-test series, the HA concentration (mg HA/L) was estimated as 2-fold the nominal DOC concentration.

5.2.5 Concentration response analysis

Differences in control reproduction between test plates in the natural DOC test series were tested with a Kruskal Wallis test in R 2.14.1 (R Development Core Team, Vienna, Austria), because the normality criterion for parametric testing was not fulfilled. Concentration response

analyses were performed on relative reproduction (RR) data. The relative reproduction of every replicate for all metal treatments was calculated with Equation 5.1. The corresponding relative effects (RE) were calculated with Equation 5.2.

$$RR_{Zn_x-Ni_y}(\%) = \frac{R_{Zn_x-Ni_y}}{R_{con}} \times 100\% \quad (5.1)$$

$$RE_{Zn_x-Ni_y}(\%) = 100 - RR_{Zn_x-Ni_y}(\%) \quad (5.2)$$

Where $RR_{Znx\text{-}Niy}$ is the relative reproduction of the treatment with Zn at concentration x and Ni at concentration y. $R_{Znx\text{-}Niy}$ is the total reproduction of the treatment with Zn at concentration x and Ni at concentration y. R_{con} is the average total reproduction of the control (i.e. the treatment where no Ni was added and Zn was present at its background concentration (i.e. ±20 µg/L); diamond in Figure 5.1 and also termed 'absolute control'). Based on the relative reproduction, a concentration response curve was fitted for every horizontal and vertical row in the full-factorial design (Figure 5.1). Each of these lines has a so-called 'mixture control' which is the treatment with only one metal added (or with background Zn; open symbols in Figure 5;1). A log-logistic concentration response curve (Eq. 5.3) was fitted to the data based on both mean measured dissolved Ni or Zn concentrations and WHAM calculated mean of Ni^{2+} and Zn^{2+} activities of the fresh and old media in STATISTICA 7 (Stat Soft Inc, Tulsa, OK, USA).

$$y = \frac{k}{1+\left(\frac{x}{EC50}\right)^{\beta}} \quad (5.3)$$

Where y is the RR (%; reproduction relative to the absolute control). k is the upper limit of y, i.e. the RR at the mixture control and EC50 is the median effective concentration: the concentration/activity ($EC50_{Mediss}$/$EC50_{Me2+}$, respectively) inducing 50% effect on *D. magna* reproduction relative to the mixture control; x is the metal concentration/activity in the test medium and β is the slope parameter. The k parameter was not fitted but fixed at the average relative reproduction (RR) of the corresponding mixture control. This k parameter is only 100% for the curves fitted on the single Zn or Ni toxicity data, not for the mixtures. The concentration response data were checked for the presence of a hormesis effect with the method described by Van Ewijk and Hoekstra (1993). The $EC50_{Me2+}$ between both tests were statistically compared using the Wheeler ratio test (2006).

5.2.6 Analysis of interactive mixture effects

As a first step in studying the interactive effects of Ni and Zn, the global interactive effects (i.e., considering the entire data of a test) in the binary 21-day reproduction toxicity test were assessed through the mixture analysis framework developed by Jonker et al. (2005) as described by Hochmuth et al. (2014). This framework allows for analyzing whether a mixture deviates from strict non-interaction using both the concentration addition (CA) and the independent action (IA) reference models. The mean relative reproduction for every Ni-Zn treatment was used as input for the mixture analysis. The analysis of the global interactive effects was performed in three steps. First, predictions of the RR for the mixture combinations were made with the reference models CA (Eq. 5.4) and IA (Eq. 5.5) assuming no interaction and using the EC50 and β of the individual concentration response curves of Ni ($EC50_{Ni}$ and β_{Ni}) and Zn ($EC50_{Zn}$ and β_{Zn}) calculated with Equation 5.3. Equation 5.4 was solved by using the generalized reduced gradient iterative solver function in Excel 2010.

$$\frac{x_{Ni}}{EC50_{Ni}\times\left(\frac{100-y}{y}\right)^{\frac{1}{\beta_{Ni}}}}+\frac{x_{Zn}}{EC50_{Zn}\times\left(\frac{100-y}{y}\right)^{\frac{1}{\beta_{Zn}}}}=1 \qquad (5.4)$$

$$y=100\times\left(\frac{1}{1+\left(\frac{x_{Ni}}{EC50_{Ni}}\right)^{\beta_{Ni}}}\right)\left(\frac{1}{1+\left(\frac{x_{Zn}}{EC50_{Zn}}\right)^{\beta_{Zn}}}\right) \qquad (5.5)$$

In the second step, the above reference models were fitted to all data (single metals and mixture data). In the third step, the CA and IA reference models were extended with a deviation parameter (*a*), which is a measure of the magnitude of the interactive effects (Jonker et al. 2005; see Hochmuth et al. 2014 for details).

For step 2 and 3, the best set of parameters (i.e. $EC50_{Me}$, β_{Me} and for step 3 *a*) was selected based on the lowest sum of squared errors of 5000 samples simultaneously taken from a normal distribution with the mean and standard deviation from the parameter values originating from the single concentration response curves. The analysis was performed in the software package R-version 2.14.1 (R Development Core Team). After checking for the validity of assumptions, an F-test was used to statistically test whether the addition of the deviation parameter *a* significantly improved the predictions of the nested models from step 2 and 3 (Asselman et al. 2013).

The results were visualized (Figure 5.3) by plotting the observed RR of the mixture treatments, together with the RR predicted by the CA and IA models in step 1 in function of the sum of toxic units of the Ni-Zn combinations (Eq. 5.6).

$$\sum TU_{Ni-Zn\,mix} = TU_{Ni} + TU_{Zn} = \frac{x_{Ni}}{EC50_{Ni}} + \frac{x_{Zn}}{EC50_{Zn}} \quad (5.6)$$

Treatment specific interactive effects were analyzed by comparing the CA and IA predicted RR for every treatment from step 1 with the observed RR of the corresponding treatment. Predicted RRs that were outside the 95% confidence intervals of the observed RR were considered as a significant deviation from non-interaction for the respective reference models (Sharma et al. 1999).

The analysis of global and treatment specific interactive effects was made based on dissolved concentrations and based on free ion activities, to identify interactions due to competitive binding of Ni and Zn onto DOC. Since free ion activities are the bioavailable fraction and therefore more relevant for toxicity than the dissolved concentrations, results of analysis based on activities are shown in the main paper and the corresponding results based on dissolved concentrations are given in Appendix B.

5.3 RESULTS

5.3.1 Concentration response analysis

Mean net control reproduction was 60.3±24.8 and 70.3±17.4 juveniles per female (±standard deviation; n=77) for the Natural DOC and AHA test series, respectively. Control mortality was 10% and 20% for the Natural DOC and AHA test series, respectively. Hence, all validation criteria of the *D. magna* reproduction test (OECD 2012) were met. There were no significant differences in control reproduction among the test plates of the Natural DOC test series (p=0.72, n=8). Dissolved Ni and Zn concentrations in the fresh medium were close to total Ni and Zn concentrations in both test series (Appendix B: Table B2). Dissolved Ni and Zn concentrations in the old solutions were a maximum 43% lower than in the fresh solutions. Median effective concentrations for the single metal exposure are summarized in Table 5.1. The $EC50_{Ni2+}$ ($EC50_{Nidiss}$) of the single Ni dose was 1035±77 nmol/L (118±8 µg/L) and 1652±358

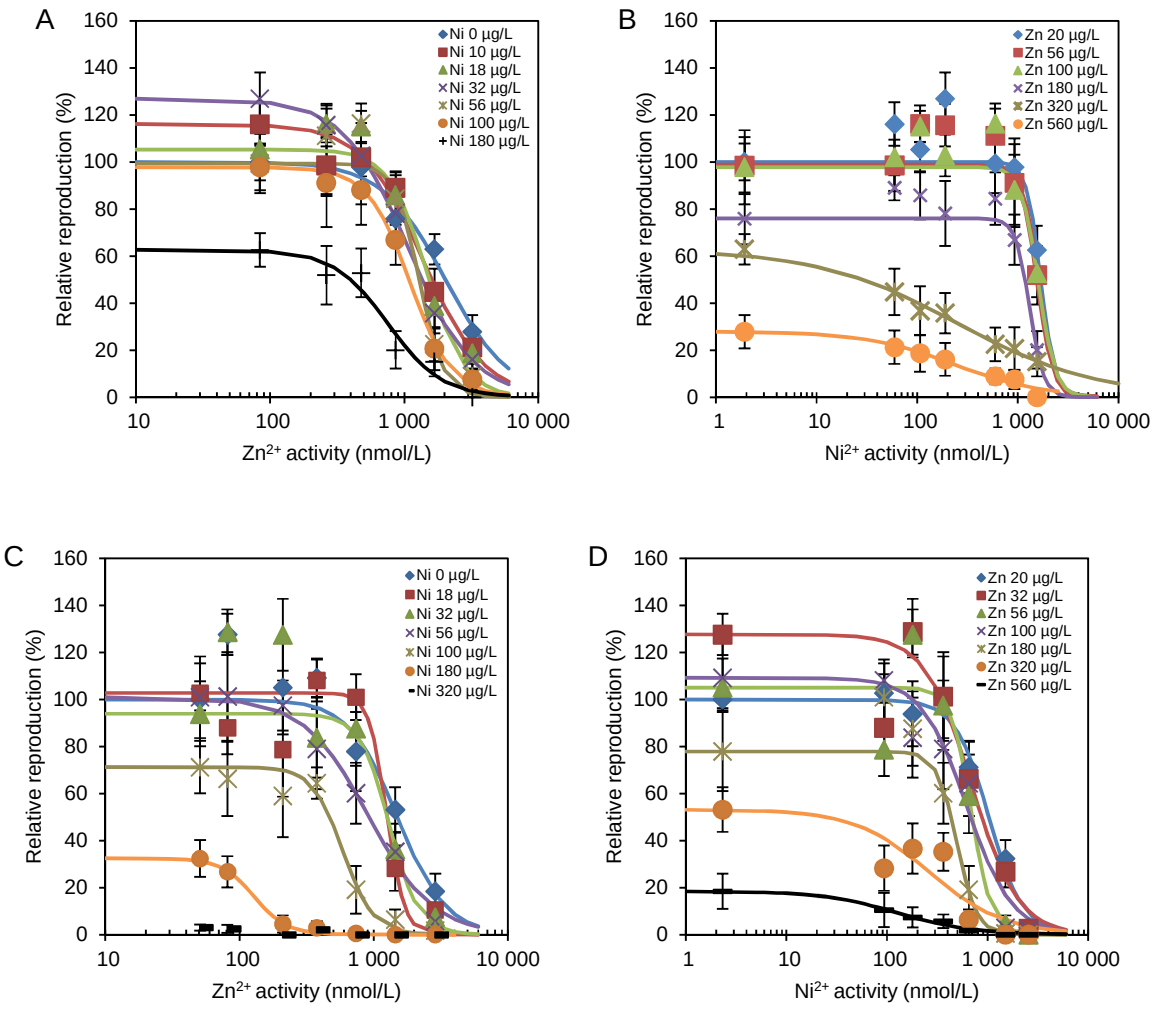

Figure 2. Concentration response data for (21d-)relative reproduction (RR; %) (symbols) of *Daphnia magna* exposed to a binary Ni-Zn mixture and fitted log-logistic concentration response curves (Eq. 5.3; lines) under different Ni treatments as a function of Zn^{2+} activities for the AHA- (A) and natural DOC-test series (C) or under different Zn treatments as a function of Ni^{2+} activities for the AHA- (B) and natural DOC-test series (D). Error bars indicate standard errors (n=10). Parameters of the concentration response curves are reported in Table B.2.

nmol/L (165±36 µg/L) for the Natural DOC and AHA test series, respectively. The $EC50_{Zn2+}$ ($EC50_{Zndiss}$) of the single Zn dose was 1496±311 nmol/L (280±48 µg/L) and 2081±166 nmol/L (264±19 µg/L) for the Natural DOC and AHA test series, respectively. The $EC50_{Ni2+}$ differed significantly between both test series (p=0.02), while this was not the case for the $EC50_{Zn2+}$. However, $EC50_{Ni2+}$ were within twofold error of each other, which is in line with standard inter-test variabilities observed for Zn (Heijerick et al. 2005) and Ni toxicity (Deleebeeck et al. 2008) to *D. magna*. Concentration response data and the fitted curves for the effect of Zn^{2+} on Ni^{2+}

toxicity and for the effect of Ni^{2+} on Zn^{2+} toxicity for both test series are shown in Figure 5.2. The corresponding figures based on dissolved concentrations are shown in Appendix (Figure B.1). Parameters of the different concentration response curves (see Eq. 5.3) are given in Appendix B (Table B.2). A significant hormesis effect was only observed for dissolved Zn toxicity at a mean dissolved Ni concentration of 25 µg/L (Figure B.A).

Table 5.1. Parameters of the concentration response curve describing single Ni and Zn (21d) reproductive toxicity to *Daphnia magna* (open symbols in Figure 5.1) based on dissolved concentrations (Mediss) and free ion activities (Me^{2+}) for both test series. Values are estimated parameters ± standard error

		Ni	Zn
Natural DOC	$EC50_{Mediss}$ (µg/L)	118±8	280±48
	β_{Mediss}	2.90±0.46	2.97±1.40
	$EC50_{Me2+}$ (nmol/L)	1035±77	1496±311
	β_{Me2+}	2.70±0.43	2.42±1.14
Aldrich Humic Acid	$EC50_{Mediss}$ (µg/L)	165±36	264±19
	β_{Mediss}	6.29±13.57	2.11±0.33
	$EC50_{Me2+}$ (nmol/L)	1652±358	2081±166
	β_{Me2+}	6.25±13.45	1.96±0.31

EC50=median effective concentration; β= slope parameter of the concentration response curve

5.3.2 Analysis of mixture effects

Figure 5.3 and Figure B.2 show the CA and IA predicted and observed RR of the mixture treatments as a function of the sum of Toxic Units (TU) based on activities and dissolved concentrations, respectively. At low TUs the CA and IA predicted RRs are relatively close to the observed RRs, while at high TUs the CA and IA predicted RRs are mostly higher than the observed RRs, i.e. suggesting synergisms at higher toxic conditions. Analysis of the global interactive mixture effects showed that the IA model extended with the parameter *a,* which tests for possible interactive effects, resulted in a negative *a* value for both test series, indicating synergistic interactions in the Ni-Zn mixture (Table B.3). Adding this parameter significantly improved the IA model fit (p=0.04 and p=0.02 for the Natural DOC- and AHA test, respectively; Figure 5.4 based on activities; Figure B.3 in Appendix B based on dissolved concentrations). For the CA reference model this *a* value was close to zero for the natural DOC test series and positive for the AHA test series, but for both test series adding this deviation parameter did not lead to a significant improved fit of the CA model (p>0.99 for both tests; Figure 5.4). For both tests the CA model fitted the data slightly better than the IA model (lower AIC; Table B.3). There

were no differences in the direction of the interactive effects when the models were fitted using either free ion activities or dissolved concentrations (Table B.3).

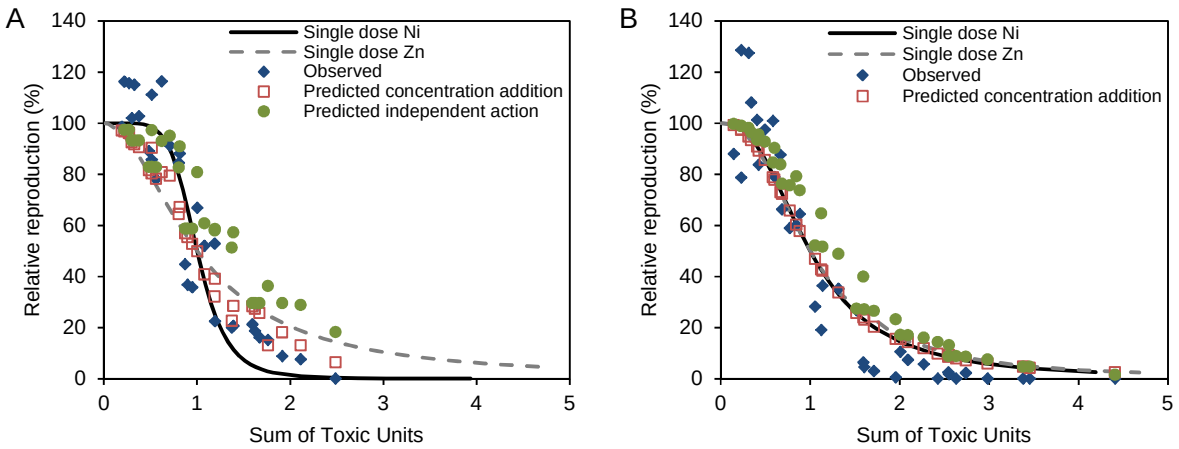

Figure 5.3. Observed and predicted (21d) relative reproduction (RR; %) of *Daphnia magna* in the mixture combinations of the Ni-Zn mixture as a function of the sum of Toxic Units (TU) based on free ion activities for the AHA-test series (A) and the natural DOC test series (B). Symbols are denoted as follows: observed effects (♦), predictions of CA (Eq. 5.4; □), predictions of IA (Eq. 5.5; ●). Predictions are based on the parameters ($EC50_{Me2+}$ and β_{Me2+}) of the individual concentration response curves of Ni and Zn (Eq. 5.3).

The interactions between Ni and Zn were clearly effect size dependent (Figure 5.5 and Figure B.4; Table 5.2 and 5.3). For the IA model, predicted RRs were lower or not significantly different compared to the observed RRs at low Ni and Zn effect sizes. This means that only non-interaction or weak antagonisms occurred in mixture treatments in which each of the individual metals had no or only weak adverse effects on reproduction on their own (i.e. minimum RR≥80%, less than 20% effect, Figure 5.5.A). On the other side of the spectrum, significant synergistic interactions started to occur when one of the two metals in the mixture combination was present at a concentration that caused at least 20% effect (i.e. minimum RR<80%). These effect size-dependent interactions become clear in Figure 5.5.A, where the ratio of observed RR to the IA-predicted RR is plotted as a function of the lowest of both RRs observed in the corresponding single metal treatments of both metals present in the mixture, i.e. the minimum RR of individual Ni and Zn treatments. As an example, for the mixture treatment Ni 44 µg/L-Zn 157 µg/L of the Natural DOC test, the RR of the corresponding single metal treatments were 101% and 78% (Table 5.2) for Ni and Zn, respectively. Consequently 78% is the minimum RR for this mixture treatment (and 22% the maximum effect size). In all treatments where both

metals caused less than 20% effect (to the right of the vertical line, i.e. minimum RR > 80%) only non-interactive and antagonistic interactions occur (only triangles and diamonds), while cases with significant synergisms start to occur at the left side of this line (Figure 5.5.A). Additionally, only significant synergistic interactions according to IA were observed when both metals were present at a concentration that individually caused 20% effect (i.e. maximum RR ≤ 80%, Figure B.4A).

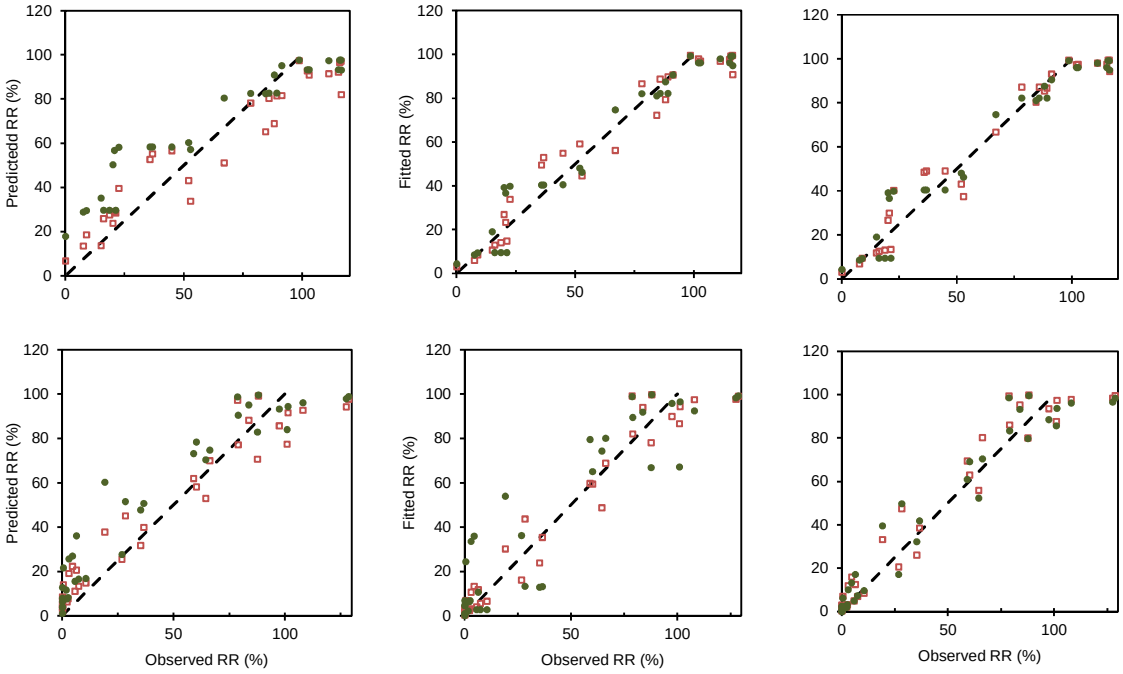

Figure 5.4. Observed relative reproduction (RR; %) versus predicted or fitted RR for the mixture reference models CA (□) and IA (●) for *Daphnia magna* after 21d-exposure to binary mixtures of Ni and Zn (only mixture data is shown) for the AHA- (upper panels) and the Natural DOC test series (lower panels). Model parameters were derived based on free ion activities. Left panel shows model predictions based on parameters of the single metal exposures, middle panels shows models fitted to all data (single metal and mixture treatments), right panel shows models extended with interactive effects parameter fitted to all data.

Similar effect size dependent interactive effects were observed for CA (Table 5.2 and Table 5.3, Figures 5.5.B and Figure B.4B), i.e. both non-interaction or antagonism at low effect mixture combinations (minimum RR>80%, maximum ≤20% effect, Figure 5.5.B) and significantly synergistic interactions at higher effect mixture combinations (maximum RR<60%, minimum ≥40% effect, Figure B.4B). The trends in the above mentioned interactions based on Ni^{2+} and Zn^{2+} activities

were similar when analysed on the basis of dissolved metal concentrations (Table B.4 and B.5, respectively).

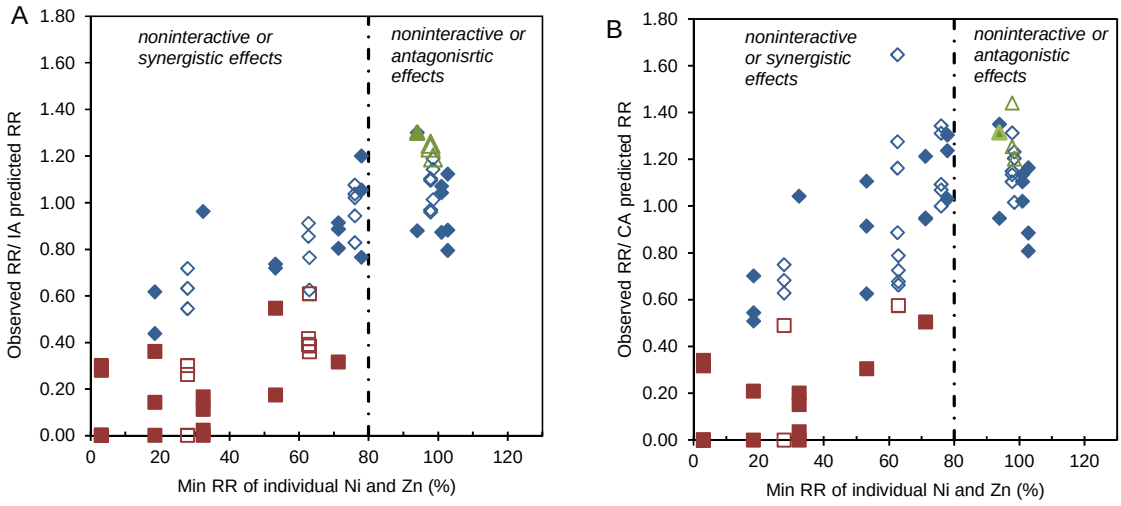

Figure 5.5. Ratio of observed and Independent Action (IA, Eq. 5.5: left panel) and Concentration Addition (CA, Eq. 5.4: right panel) predicted 21d-relative reproduction (RR, %) of *Daphnia magna* in the mixture treatments as a function of the minimal RR in the corresponding single dose treatments of Ni and Zn. Values higher than 1 indicate possible antagonistic interactions, values lower than 1 indicate possible synergistic interactions. Symbols are denoted as follows: significant antagonistic interactions (▲), significant synergistic interactions (■) and non-interaction (♦). Filled symbols denote treatments from the natural DOC series, open symbols from the AHA test series

Table 5.2. Mean observed relative reproduction (RR; %) for *Daphnia magna* during 21d exposure to combinations of Ni and Zn for the Natural DOC test series. 95% confidence intervals on RR are reported between brackets. Predicted RR based activities (IA/CA) are reported in italics.

	Zn 17 µg/L[a]	**Zn 25 µg/L**	**Zn 57 µg/L**	**Zn 92 µg/L**	**Zn 157 µg/L**	**Zn 271µg/L**	**Zn 478 µg/L**
Ni 0.7 µg/L	100 (91-109)	128 (108-148)	105 (60-150)	109 (91-128)	78 (40 112)	53 (32-75)	18 (1-35)
Ni 14 µg/L	103 (74-131)	88 (62-114) *100/99 (0/0)[b]*	79 (33-124) *99/97 (0/0)*	108 (88-128) *96/93 (0/0)*	101 (79-123) *84/77 (0/0)*	28 (6-50) *52/45 (+/0)*	10 (0-27) *17/15 (0/0)*
Ni 25 µg/L	94 (62-126)	129 (107-150) *99/98(-/-)*	127 (93-162) *98/94 (0/0)*	84 (44-123) *95/88 (0/0)*	88 (50-125) *83/71 (0/0)*	37 (12-61) *51/40 (0/0)*	7 (2-17) *17/14 (0/0)*
Ni 44 µg/L	101 (62-140)	101 (59-144) *95/92 (0/0)*	97 (73-121) *93/86 (0/0)*	79 (40-118) *91/77 (0/0)*	60 (30-90) *78/58 (0/0)*	*35 (17-54)* 48/32 (0/0)	6 (0-12) *16/11 (+/0)*
Ni 80 µg/L	71 (46-96)	66 (30-102) *75/70 (0/0)*	59 (19-99) *73/62 (0/0)*	64 (49-79) *71/59 (0/0)*	20 (0-42) *60/38 (+/0)*	*6 (0-16)* 36/21 (+/+)	2 (0-5) *12/8 (+/+)*
Ni 163 µg/L	32 (15-50)	27 (12-42) *28/26 (0/0)*	4 (0-13) *27/22 (+/+)*	3 (0-9) *26/19 (+/+)*	0 (0-1) *22/14 (+/+)*	0 *13/9 (+/+)*	0 *4/4 (+/+)*
Ni 264 µg/L	3 (0-6)	2 (0-6) *8/8 (+/+)*	0 *8/7 (+/+)*	2 (2-6) *8/6 (+/+)*	0 *7/5 (+/+)*	0 *4/4 (+/+)*	0 *1/2 (+/+)*

[a] Reported concentrations are the mean of measured dissolved Ni and Zn concentrations in fresh and old medium

[b] Deviations from non-interaction are reported between brackets; 0 non-interaction, + observed joint effect higher than predicted, - observed joint effect lower than predicted

Table 5.3. Mean observed Relative Reproduction (RR; %) for *Daphnia magna* during 21d exposure to combinations of Ni and Zn for the AHA test series. 95% confidence intervals on RR are reported between brackets. Predicted RR based on activities (IA/CA) are reported in italics.

	Zn 21 µg/L[a]	Zn 48 µg/L	Zn 77 µg/L	Zn 126 µg/L	Zn 223 µg/L	Zn 399 µg/L
Ni 0 µg/L	100 (82-118)	98 (71-126)	98 (62-133)	76 (51-101)	63 (48-78)	28 (12-44)
Ni 6 µg/L	116 (95-137)	99 (71-126) *97/97 (0/0)*[b]	102 (69-135) *93/92 (0/0)*	89 (77-101) *83/81 (0/0)*	45 (22-67) *59/57 (0/0)*	21 (5-37) *30/28 (0/0)*
Ni 11 µg/L	105 (83-127)	116 (98-134) 97/97 (-/-)	115 (100-130) *93/92 (-/-)*	86 (63-109) *83/80 (0/0)*	37 (13-60) *59/55 (0/0)*	19 (1-36) *30/27 (0/0)*
Ni 19 µg/L	127 (102-152)	116 (95-136) *97/97 (0/0)*	103 (83-123) *93/91 (0/0)*	78 (47-109) *83/78 (0/0)*	36 (16-55) *59/53 (+/0)*	16 (0-32) *30/26 (0/0)*
Ni 59 µg/L	99 (71-128)	111 (85-137) *97/90 (0/0)*	116 (97-135) *93/81 (-/-)*	84 (59-110) *83/64 (0/0)*	22 (6-39) *59/39 (+/+)*	9 (1-16) *29/18 (+/+)*
Ni 91 µg/L	98 (76-120)	91 (48-134) *95/79 (0/0)*	88 (54-122) *91/67 (0/0)*	67 (43-91) *81/50 (0/0)*	21 (0-41) *57/28 (+/0)*	8 (0-17) *29/13 (+/0)*
Ni 152 µg/L	63 (39-86)	52 (24-80) *61/41 (0/0)*	53 (43-91) *58/32 (0/0)*	20 (2-38) *51/23 (+/0)*	15 (1-29) *36/13 (+/0)*	0 *18/6 (+/+)*

[a] Reported concentrations are the mean of measured dissolved Ni and Zn concentrations in fresh and old medium

[b] Deviations from non-interaction are reported between brackets; 0 non-interaction, + observed joint effect higher than predicted, - observed joint effect lower than predicted

5.4 DISCUSSION

We investigated the interactive effects of a binary Ni-Zn mixture on reproductive toxicity in *D. magna*, using two independent large-scale experiments. The analysis of the global interactive effects in the binary Ni-Zn mixtures indicated significant synergistic interactions between Ni and Zn in both test series using the IA model, while there were no global interactive effects between Ni and Zn according to the CA model. However, analysis at the treatment level showed that the occurrence and the direction of the interactive effects for both the CA and IA model were highly dependent on the effect size at which both metals were combined in the mixture. In general, non-interactive or antagonistic effects were observed at low effect sizes of individual Ni and Zn (maximum 20% effect) for both reference models and in both test series. However, when Ni and Zn concentrations induced a certain threshold level of toxicity on their own, synergistic interactions started to occur for both reference models. A significant synergistic effect according to the IA model was always observed when both metals were present at a concentration individually inducing approximately 20% effect or more. For the CA model, synergistic mixture effects occurred in all mixture treatments where both metals in the mixture already caused a greater than 40% effect on reproduction on their own. This suggests that this threshold level of

toxicity above which IA and CA synergistic effects on reproductive toxicity to *D. magna* occur in Ni and Zn mixtures is situated around the EC20 and EC40 of the individual metals, respectively.

The reproducibility of concentration dependent interactive effects has been debated (Cedergreen et al. 2007). However, our study shows very similar patterns in two independent data sets. Similar shifts from non-interactive or antagonistic effects at low concentrations to synergistic effects at concentrations above a certain threshold have been observed for different binary metal mixtures (e.g. Cu-Cd, Zn-Cd, Cu-Zn) and within different taxonomic groups, e.g. protozoa (Galeggo et al. 2007), plants (Sharma et al. 1999) and marine algae (Wang et al. 1995).

The mainly synergistic toxic effects at high Ni and Zn effect concentrations were initially unexpected (see Introduction), as Ni and Zn show competitive interaction (antagonisms) for metal uptake during short-time exposures with *D. magna* (Komjorova & Blust 2008) and several other species (Fu & Maier 1991; Komjorova & Blust 2009; Worms & Wilkinson 2007; Israr et al. 2011). In contradiction, Bourgeault et al. (2012) reported that the uptake of Ni by the freshwater mussel *Dreissena polymorpha* was enhanced in the presence of elevated concentrations of Zn. This confirms that the direction of metal interaction can be dependent on the tested species (Norwood et al. 2003). Several possible explanations exist for the observed inconsistency in the direction of interactive effects of Ni and Zn between the level of uptake by and toxicity to *D. magna*. First, the direction of metal mixture interactions can vary substantially depending on exposure concentrations (see above; Sharma et al. 1991; Norwood et al. 2003). Therefore, we calculated Ni^{2+} and Zn^{2+} activities of the exposure concentrations in the *D. magna* uptake study (Komjarova & Blust 2008) based on the reported nominal chemistry of the test medium, assuming that deionized water contains 0.3 mg/L of DOC (Van Sprang et al. 2009) and DIC concentration was calculated based on the reported pH (5.18E-5 mol DIC/L; Van Sprang et al. 2009). The same assumptions were made as those used for speciation calculations in the Ni-Zn mixtures of the present study (Section 2.4). Ni^{2+} (65-855 nmol/L) and Zn^{2+} (57-810 nmol/L) activity ranges used in the exposures of the accumulation study of Komjarova and Blust (2008) were similar to the low and intermediate Ni and Zn concentrations treatments applied in the present study (Table B.2). Although the non-interactive and antagonistic interactions of Ni and Zn at low effect combinations observed in the present study are in agreement with the study of Komjorova and Blust (2008), synergistic effects of Ni and Zn on reproductive toxicity were already observed at the intermediate activities in the present study. Hence, differences in

exposure concentrations are probably not an explanation for the difference in reported metal mixture interactions between uptake and toxicity. Other possible explanations are differences in exposure time (chronic vs. short-time; Cooper et al. 2009) and exposed life stage (juveniles vs. adult daphnids; Zhu et al. 2011). However, interactive effects on the level of accumulation during exposure to metal mixtures do not necessarily reflect toxicity effects (Komjarova & Blust 2008).

The competing effects between Ni and Zn for uptake is often explained as competition for a common uptake site (Fu & Maier 1991; Komjorova & Blust 2008; Worms & Wilkinson 2007; Israr et al. 2011). However, Komjarova and Blust (2009) hypothesized that the decrease in Ni uptake by the Zebrafish *Danio rerio* in the presence of Zn could be the result of the regulation of metal transferring proteins by Zn, since Ni and Zn are known to interfere with different uptake pathways of major ions (e.g. for *D. magna:* the Mg uptake pathway for Ni (Pane et al. 2003) and the Ca uptake pathway for Zn (Muyssen et al. 2006)). A Zn induced regulation of metal transferring proteins was previously also suggested to explain the observed synergistic effect of Zn on Ni uptake by *D. polymorpha* (Bourgeault et al. 2012). Similarly, the enhanced uptake of Pb at low ambient concentrations of Cu by the green alga *Chlamydomonas reinhardtii* was linked to an increased expression of *ctr2*, a gene which codes for a high-affinity Cu transporter (Chen et al. 2010). A similar metal induced regulation of metal transferring proteins may have resulted in the observed synergistic effect on toxicity to the binary Ni-Zn mixture. However, synergistic interactions may, if both metals rely on the same cellular detoxification system, also result from a saturation of detoxification mechanisms (Sharma et al. 1999), or from a degradation of the cell membrane permeability (Babich & Stotzky 1983). In general, it can be concluded that there is a need to mechanistically (i.e. on the molecular and physiological level) understand the observed interactions.

The analysis of interactive mixture effects showed similar results when the models were fitted based on dissolved concentrations or on calculated activities. This indicates that competition effects due to DOC did not influence the nature of the interactive effects. This is important because competition between metals for binding on the DOC molecules may potentially cause apparent synergistic effects at the level of dissolved concentrations, while this is not the case at the actual bioavailable (free ion level) and thus toxic fraction. In addition the ability to protect against metal toxicity varies considerably between different types of DOC (Wood et al. 2011). As a result the types of interactive effect observed based on dissolved concentrations could

potentially be different between media with DOM from different origins. Hence, it is important to analyze metal mixture interactions not only on the basis of dissolved concentrations, but also on the basis of free ion activities, which can either be modeled by speciation software or directly measured in the exposure media.

In the environment, metals generally occur as mixtures. To date, risk assessment mainly focusses on single substances, while mixture toxicity is rarely considered (Backhaus & Faust 2012). The incorporation of mixture toxicity in risk assessment processes is, however, unavoidable in the near future (CEU 2009). Recently, the CA model was proposed as a first tier approach for the risk assessment of mixtures (Backhaus & Faust 2012), since the CA model in general provides more conservative predictions for mixture toxicity than IA (e.g. Altenburger et al. 1996; Cedergreen et al. 2008). Indeed, also for this dataset the CA model is mostly more conservative than the IA model (Figure 5.3). Furthermore, the CA model and the IA model appear to be protective for most Ni-Zn combinations in the low effect size range. In contrast, due to the synergisms that became apparent at combinations of high Ni and Zn concentrations both models did not appear to be protective when Ni and Zn co-occur in high concentrations, which may appear under local contamination situations. However, since mainly low effect sizes are relevant for environmental risk assessment (EC 2003), the CA and IA model can both serve as a protective scenario for the risk assessment of Ni and Zn mixtures for *D. magna* reproduction. The applicability of this approach for other species and other metal combinations remains to be tested, since the direction and magnitude of interactive effects may differ depending on the tested species and metal combination (Norwood et al. 2003).

An additional complicating factor in the risk assessment of metals and metal mixtures, is that the bioavailability and therefore also the toxicity of metals is highly dependent on the physicochemical composition of the receiving surface water (pH, DOC, hardness,…) (e.g., De Schamphelaere & Janssen 2004b; Heijerick et al. 2005; Grosell et al. 2006a; Deleebeeck et al. 2008). In the past decade, bioavailability models, such as the Biotic Ligand Model (BLM) (e.g. Heijerick et al. 2005; Deleebeeck et al. 2008), have been developed that are able to predict individual chronic metal toxicity in a specific surface water. These models relate metal toxicity to the concentration of metal binding at the biotic ligand (BL), i.e. cell surface receptor, and the activity of specific cations (e.g., $Ca^{2+,}$ H^{+} , Mg^{2+}), which compete with the metal for binding at the BL (Di Toro et al. 2001). BLMs are currently increasingly being incorporated in risk assessment

procedures for individual metals in Europe (Van Sprang et al. 2009) and the United States (USEPA 2007). BLMs can theoretically also be used to predict the toxicity of metal mixtures (Di Toro et al. 2001). So far, only a limited number of studies have considered the BLM approach to model toxicity of metal mixtures (Playle 2004; Hatano & Shoji 2008; Kamo & Nagau 2008; Jho et al. 2011; Le et al. 2013; Balistrieri & Mebane 2014; Versieren et al. 2014). However, most of these metal mixture studies considered acute toxicity (e.g. Hatano & Shoji 2008; Kamo & Nagau 2008; Jho et al. 2011; Le et al. 2013; Versieren et al. 2014), while the application of the BLM concept to chronic mixture toxicity data remains to be tested.

Antagonistic interactions between metals can easily be explained and modeled in the BLM concept as a competition effect of one metal on the BL of another metal (e.g. Jho et al. 2011; Versieren et al. 2014). The integration of synergistic effects in the BLM, on the contrary, is from a conceptual point of view less straightforward. This is certainly the case when using the CA model, since a similar mode of action implicates a single type of BL which is shared between the different metals. An alternative way to model metal mixture toxicity is to combine the BLM approach with the IA model (Versieren et al. 2014). For the IA-BLM approach, it is assumed that each metal has a separate type of BL with different binding characteristics. Synergistic interactions can theoretically be integrated into the model structure of the IA-BLMs, by adding a model term which describes the synergistic interaction at the BL to the model term describing the intrinsic sensitivity (i.e. the hypothetical $EC50_{Me2+}$ when no competing ions are present). However, the application of this approach remains to be tested.

6

Reproductive toxicity of binary and ternary mixture combinations of Ni, Zn, and Pb to *Ceriodaphnia dubia* is best predicted with the independent action model.

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6. Reproductive toxicity of binary and ternary mixture combinations of Ni, Zn, and Pb to *Ceriodaphnia dubia* is best predicted with the independent action model

6.1 Introduction

Although metals occur as mixtures in the environment, risk assessment is still performed on an individual metal basis and is based on toxicity tests using single metal exposures. To be able to incorporate metal mixture toxicity in risk assessment procedures, a better understanding of metal mixture effects is needed (Meyer et al. 2015a). The following two reference models are the most widely accepted ones for evaluating mixture toxicity: Concentration Addition (CA) and Independent Action (IA) (Jonker et al. 2005). The CA model assumes that substances have the same mode of action and that the components in a mixture can be replaced with other chemicals without changing the overall mixture effects, as long as the sum of toxic units does not change. The IA model assumes that substances have a different mode of action and that the joint response to a mixture can be calculated as the product of the responses to each of the individual components in the mixture. The basic assumption of both models is that substances do not interact. However, synergistic interactions can occur if the observed joint effects are significantly larger than those predicted with these models based on the individual toxicity of the mixture components. In contrast, antagonistic interactions occur if the observed joint effects are significantly smaller than those predicted with these models.

The combined effects of metal mixtures are typically diverse, i.e. both non-interactive, synergistic, and antagonistic effects are observed irrespective of using either IA or CA as reference models (Norwood et al. 2003; Vijver et al. 2011). The type of interactive effect observed can differ among species and metal combinations (Norwood et al; 2003), can be effect-size dependent (Chapter 5) or can change with abiotic conditions, e.g. water hardness (Versieren et al. 2015). The lack of general patterns emerging from metal mixture studies currently hinders the development of models to predict the toxicity of metal mixtures (Meyer et al. 2015a). Furthermore, the majority of studies investigated the toxicity of metal mixtures during short-term, acute exposures, while many risk assessment frameworks typically require models predicting metal mixture toxicity during chronic exposures (Meyer et al. 2015a; Van Genderen et al. 2015)

To develop chronic metal mixture models, we need to understand how metals interact in metal mixtures during chronic exposure. The incorporation of mixture toxicity – i.e. the choice of either CA or IA - is crucial for future risk assessment procedures. Predictions based on the CA model will be different from those of the IA model and will lead in most cases to more stringent metal water quality criteria. A priori knowledge of modes of action may be used to select either IA and CA in those risk assessment procedures. Therefore, we investigated the interactive effects in all possible binary and ternary mixture combinations of Ni, Zn, and Pb (i.e. Ni-Zn, Pb-Zn, Ni-Pb, and Ni-Zn-Pb) using the chronic *Ceriodaphnia dubia* 7d-reproduction test. These three metals were selected because different ions have been shown to compete with these metals at uptake sites, which suggests the occurrence of dissimilar modes of action for these metals. For example, Zn toxicity to daphnids has been related to a disturbed Ca balance (Muyssen et al. 2006) and solution Ca^{2+} protects against Zn toxicity (Heijerick et al. 2005; Chapter 4). Nickel toxicity has been shown to influence the Mg homeostasis (Pane et al. 2003) and Mg^{2+}/Ni^{2+} interactions in solution are relatively important for Ni toxicity (Deleebeeck et al. 2008). In contrast, neither Ca nor Mg exert a major influence on chronic Pb toxicity to *C. dubia* (Mager et al. 2011a; Chapter 2), suggesting Pb affects other targets within these organisms. Given the suspected different modes of action of these metals, we hypothesized that the toxicity of their binary and ternary mixtures follows the IA model rather than the CA model.

6.2 Materials & methods

6.2.1 Test design and test medium

The interactive effects of Ni, Zn, and Pb in binary and ternary mixture combinations were investigated in three consecutive *C. dubia* 7d-reproduction tests, denoted as Experiments (Exp.) 1, 2 and 3. All toxicity tests were conducted in modified natural water collected from L'Ourthe Oriental in Brisy, Belgium. This unpolluted water has a low hardness and Dissolved Organic Carbon (DOC) concentration and has previously successfully been used for ecotoxicity testing in our laboratory (e.g. Deleebeeck et al. 2008; Chapter 2 & 4). The natural water was filtered (0.2 µm) on site and collected in acid-washed polyethylene barrels. The barrels were stored at 4°C in total darkness upon arrival in the laboratory. The natural water used for the present study was sampled at two different occasions, the water chemistry hence varied slightly between the

different experiments. Water for the binary Ni-Zn (Exp. 1) and Pb-Zn (Exp. 2) tests was sampled in February 2014. The pH of the Brisy water at that time was 6.9 and the corresponding dissolved Ca, Mg and DOC concentrations were 10.0, 3.9 and 3.2 mg/L, respectively.. Water for the Ni-Pb and Ni-Pb-Zn test (Exp. 3) was sampled in August 2014; the pH was 7.7 and the corresponding dissolved Ca, Mg and DOC concentrations were 8.9, 3.4 and 5.7 mg/L, respectively. Background metal concentrations are reported in Appenidx D. To all test waters 0.75 mM $CaCl_2.2H_2O$ was added, as the low Ca concentrations in the Brisy water might induce physiological Ca stress (Chapter 2).

The binary mixture combinations (Zn-Ni, Zn-Pb, and Ni-Pb) were tested in three separate mixture experiments using a full factorial test design: one for every binary combination and the ternary mixture was included in the Ni-Pb experiment. Each full-factorial binary mixture test combined 7 concentrations of both metals, accordingly 49 (7x7) metal combinations were tested for every binary mixture and an additional set of 6 ternary metal combinations in Exp. 3 (Figure C1 in Appendix C). The single metal concentration treatments were always included in this full factorial design (i.e. they were tested simultaneously) and were always tested at the natural background concentrations of the other metals. For the Zn-Ni mixture, nominal added concentrations ranged from 10 to 100 µg/L for Ni and from 20 to 320 µg/L for Zn. For the Pb-Zn mixture, nominal added concentrations ranged from 30 to 260 µg/L for Pb and from 20 to 320 µg/L for Zn. For the Ni-Pb mixture, nominal added concentrations ranged from 5 to 90 µg/L for Ni and from 100 to 500 µg/L for Pb. Concurrently with the binary Ni-Pb test the single dose-response of Zn (range 20-320 µg nominal added Zn/L) and toxicity of the ternary Ni-Zn-Pb mixture was tested using an equitoxic ray design (Figure C.1D in Appendix C). Ni-Zn-Pb equitoxic mixture combinations were tested at 0.1, 0.2, 0.5, 1.0, 2.0 and 3.0 sum of Toxic Units (TU). TU was defined as the concentration of metal *i* (c_{Me_i}) divided by the 50% effective concentration of metal *i* ($EC50_{Me_i}$; Equation 6.1). The individual-metal EC50 values used to select Ni, Pb, and Zn concentrations for the equitoxic ternary mixture combinations in Experiment 3 were obtained from results of Experiments 1 and 2.

$$TU_i = \frac{c_{Me_i}}{EC50_{Me_i}} \quad (6.1)$$

The treatment combinations were prepared by adding $ZnCl_2$, $NiCl_2$, and $PbCl_2$ (analytical grade). All chemicals were purchased from VWR International.

6.2.2 Ecotoxicity testing

The chronic mixture toxicity tests with *C. dubia* were conducted following the USEPA standard test protocol (USEPA 2002a). *C. dubia* juveniles originated from an in-house isoclonal laboratory culture which has been maintained for more than 20 years at 25°C in activated carbon-filtered Ghent city tap water to which selenium (1 µg Se/L) and vitamins (75 µg/L thiamine, 1 µg/L cyanocobalamine, 0.75 µg/L biotine) are added. Daphnids were individually acclimated to the test medium for one generation prior to test initiation (1 week) in polyethylene cups with 20 mL of the test medium (1 daphnid per cup). During the acclimation period media were renewed three times a week. During the acclimation and testing period, daphnids were daily fed with *P. subcapitata* algae (2.10^5 cells/mL) and a Yeast-Urtica-Trout Chow mixture (12 mg solids/L). Tests were initiated with juveniles from the third brood from mothers that produced at least 8 juveniles in this third brood and which had produced at least 20 juveniles during the first three broods (USEPA 2002a). Tests were conducted in polyethylene cups containing 20 mL of the test medium at 25°C and a 16h light and 8h dark cycle. Test cups were placed in holding plates that can accommodate up to 7 rows of 10 cups. Every treatment was replicated 10 times, except the non-metal amended control water (see further). The 10 replicates of every treatment were split into two series of each 5 replicates and randomly distributed over the holding plates. Additionally every holding plate also had a row of controls (modified Brisy medium without extra metals added), to account for possible plate differences in control reproduction. Treatments were distributed over 8 plates (Exps. 1 & 2) or 10 plates (Exp. 3) and 80 controls were tested in Exps. 1 & 2 and 100 in Exp. 3. Juveniles (<24h old, 1 per replicate) were distributed among treatments as described in the USEPA protocol (USEPA 2002a). Test waters were completely renewed daily. Before renewal, fresh test waters were, if needed, adjusted to the required pH by adding dilute HCl or NaOH. Mortality and number of juveniles were scored daily. Toxicity tests were ended when 60% of the control animals had produced three broods.

6.2.3 Analytical chemistry

During the test period, samples of fresh (sample of new medium just before transfer of daphnids to the cup) and old (sample taken of medium just after transfer of daphnids to a new cup) test waters of all treatments were collected regularly for analysis of total and filtered (0.45 µM, Acrodisc, PALL Life Sciences) metals. Samples for analysis of total and dissolved organic carbon

and inorganic carbon were collected for random selected treatments. Total and filtered samples of fresh test waters were taken on days 0 and 6. Filtered samples of old test waters were taken on days 1 and 7. Samples for metal analysis were acidified to 0.14 mol/L HNO_3 (Normatom quality, VWR Prolabo). Nickel and Pb concentrations were measured using Graphite Furnace Atomic Absorption Spectrophotometry (GFAAS Furnace Autosampler, Thermo Fisher Scientific Inc., Reference Material TM-24.3, lot 0510 [Environment Canada]: limit of quantification 1 µg Ni/L and 1.4 µg Pb/L , method detection limit 0.3 µg Ni/L and 0.4 µg Pb/L). Zinc concentrations were measured using Flame Atomic Absorption Spectrophotometry (SpectrAA100, Varian; Reference Material TMDA-70, lot 0310 [Environment Canada]: limit of quantification 20 µg Zn/L, method detection limit 10 µg Zn/L). When measured concentrations were below the limit of quantification, the limit of quantification divided by two was used as the concentration input for further analyses. The DOC and DIC (Dissolved Inorganic Carbon) were measured with a Total Organic Carbon analyser following the NPOC method (TOC-5000, Shimadzu, Duisburg, Germany; Limit of Quantification 1.5 mg DOC/L; Method Detection Limit 0.5 mg DOC/L). Samples of Na, Ca, Mg, and K were taken at the start of each test, and were measured using Inductively Coupled Plasma Optical Emission Spectroscopy (Perkin Elmer 3300 DV). Chloride and sulphate samples were taken at the start of each test, and were measured using UV-VIS spectrophotometry (Aquamate, Thermo Electron Corporation; Chloride: Merck, Spectroquant 1.14897.001; Sulphate: Merck, Spectroquant 1.14548.001). The pH of fresh and old test waters was measured daily with a glass electrode (P407; Consort).

6.2.4 Speciation calculations

Speciation of Ni, Zn, Pb, and other major ions was calculated using the software package WHAM (Windermere Humic Aqueuos Model) VII (Tipping et al. 2015). The default stability constants for inorganic carbonate-complexes of Ni, Pb and Zn in WHAM VII were adapted to those proposed by the National Institute of Standards and Technology (Smith et al. 2004). We assumed that Dissolved Organic Matter (DOM) contains 50% carbon on a weight basis (Ritchie & Perdue 2003). Furthermore, for the DOM present in Brisy water, we assumed 65% to be active and behaving as isolated FA. Previous research has shown that assumptions between 60% and 70% active FA typically work best for predicting metal speciation in natural waters (Tipping 2002). Accordingly, the measured DOC content (mg C/L) was multiplied by a factor of 1.3 to obtain

the concentration of FA (mg FA/L) used as the modelling input. Furthermore, it was assumed that activities of the metal cations Fe^{3+} are controlled by colloidal $Fe(OH)_3$ precipitates (Lofts & Tipping 2011).

6.2.5 Dose-response analysis

Inter-test plate differences in control reproduction were evaluated with the Kruskal-Wallis test in R 2.1.4.1 (R Development Core Team). Dose-response analyses were performed on relative reproduction (RR) data, i.e. reproduction was expressed relative to the average control reproduction. The RR was calculated using Equation 6.2. The corresponding relative effects were calculated using Equation 3.

$$RR_{i,j}(\%) = \frac{R_{i,j}}{R_{con,j}} \times 100\% \quad (6.2)$$

$$RE_{i,j}(\%) = 100 - RR_{i,j}(\%) \quad (6.3)$$

Where $RR_{i,j}$ is the relative reproduction of the treatment *i* of Exp. *j*. $R_{i,j}$ is the total reproduction of the treatment *i* of Exp. *j*. $R_{con,j}$ is the average total reproduction of the control of Exp. *j* (i.e. the treatment where no Ni, Zn, or Pb was added to modified Brisy medium). Based on the relative reproduction, two parameter log-logistic dose-response curves were fitted using Equation 6.4. The dose-response curves were fitted to the data both based on dissolved concentrations (mean of concentration measured in fresh and old test waters) and on WHAM calculated free Ni^{2+}, Zn^{2+}, and/or Pb^{2+} activities in Statistica (StatSoft). A dose-response curve was fitted for every single metal concentration treatment, i.e. the first row or first column of the full factorial design in Figure C.1A-C, or the non-purple markers in Figure C.1D in Appendix C).

$$RR_i(\%) = \frac{100}{1+\left(\frac{x_{Me_i}}{\mathrm{EC50}_{Me_i}}\right)^{\beta_{Me_i}}} \quad (6.4)$$

In Equation 6.4, x_{Me_i} is the concentration/activity of metal *i* in the test medium. $EC50_{Me_i}$ is the 50% effective concentration of metal *i*, or the concentration/activity ($EC50_{Me_i diss}/EC50_{Me_i^{2+}}$, respectively) inducing a 50% effect on *C. dubia* reproduction. β_{Me_i} is the slope parameter.

6.2.6 Analysis of interactive effects

The interactive effects of Zn, Ni, and Pb were evaluated using the mixture analysis framework developed by Jonker et al. (2005) following the methodology described by Hochmuth et al. (2014). This frameworks allows to evaluate if the observed mixture toxicity, i.e. RR deviates from the RR predicted assuming strict non-interaction relative to both the CA and IA models using Equation 6.5 and Equation 6.6, respectively.

$$\sum_{i=1}^{n} \frac{x_{Me_i}}{EC50_{Me_i} \times \left(\frac{100-RR_{mix}}{RR_{mix}}\right)^{\frac{1}{\beta}Me_i}} = 1 \quad (6.5)$$

$$RR_{mix} = 100 \times \prod_{i=1}^{n} \left(\frac{1}{1+\left(\frac{x_{Me_i}}{EC50_{Me_i}}\right)^{\beta Me_i}} \right) \quad (6.6)$$

Where RR_{mix} is the predicted RR (%) of the mixture; *n* is the number of components in the mixture.

The actual analysis was performed in three steps. In the first step, metal mixture toxicity (RR) was predicted based on the parameters of the single metal dose-response curves ($EC50_{Me_i}, and\ \beta_{Me_i}$ calculated with Equation 6.4) using the above reference models. RR_{mix} in the non-linear relationship of the CA model (Eq. 6.5) was calculated iteratively using the Solver function in Excel. In the second step, Equation 6.5 and 6.6 were fitted to all data, both the single metal treatments and the mixture treatments. In the third step, the reference models were extended with the deviation parameter *a*, which is a measure for the magnitude of potential interactive effects and thus tests for deviations of non-interactivity (Jonker et al. 2005; methodology described in Hochmuth et al. 2014).

In order to solve step 2 and 3, 5000 random parameter sets (i.e. $EC50_{Me_i}, and\ \beta_{Me_i}$) were simultaneously taken from a normal distribution with the mean and standard deviation originating from the single metal dose-response curves. From these 5000 parameter sets, the best set of parameters was then selected based on the lowest sum of squared errors. After checking for the validity of assumptions, an F-test was used to evaluate if the addition of the deviation parameter *a* in step 3 resulted in a significantly better model fit compared to the model of step 2 (Asselman et al. 2013). Overall model fits were compared using the Aikake Information Criterion (AIC) corrected for the sample size. The analysis was performed for every binary or ternary mixture combination separately in the software Package R 3.1.10. The mean

relative reproduction of all treatments was used as input. Since the type of interactive effects observed can shift depending on whether the mixture analysis is based on dissolved metal concentrations or free ion activities (e.g. Meyer et al. 2015b), analyses were made for both expressions of exposure concentrations. Since the free ion represents the bioavailable metal, data for the free ion activities are given in the main paper, those for the dissolved concentrations are given in Appendix C.

6.3 Results

6.3.1 Dose-response analysis

The measured water chemistry parameters in the different mixture experiments are summarized in Table 6.1. In all experiments the validity criteria for control performance according to the standard test protocol (USEPA 2002a) were met (Table C.1 in Appendix C). No significant differences in control reproduction among holding plates were observed (Table C.1). Median effective concentrations for the single-metal exposures in each of the experiments are summarized in Table 6.2. The $EC50_{Ni2+}$ ($EC50_{Nidiss}$) in the Ni only exposures ranged between 478-546 nmol/L (43-52 µg/L; n=2). The $EC50_{Zn2+}$ ($EC50_{Zndiss}$) in the Zn only exposures ranged between 1290-2014 nmol/L (197-239 µg/L; n=3). The $EC50_{Pb2+}$ ($EC50_{Pbdiss}$) in the Pb only exposures ranged between 20-36 nmol/L (111-302 µg/L; n=2). The $EC50_{Me2+}$ were within a 2-fold error of each other, which is in line with earlier-reported inter-test variability of chronic Zn, Ni, and Pb toxicity to daphnids (De Schamphelaere et al. 2006; Chapter 2 & 4). Therefore, difference in Me^{2+} toxicity between tests may be primarily related to inter-test variability rather than differences in water chemistry. The dose-response data of the full-factorial designs and fitted dose-response curves of the individual metal exposures are shown in Figure 6.1 and Figure C.2-3 for the analysis based on free ion activities and dissolved concentrations, respectively. The EC10, EC20, EC50 and their respective 95% confidence intervals are listed in the Appendix (Table C.2 in Appendix C).

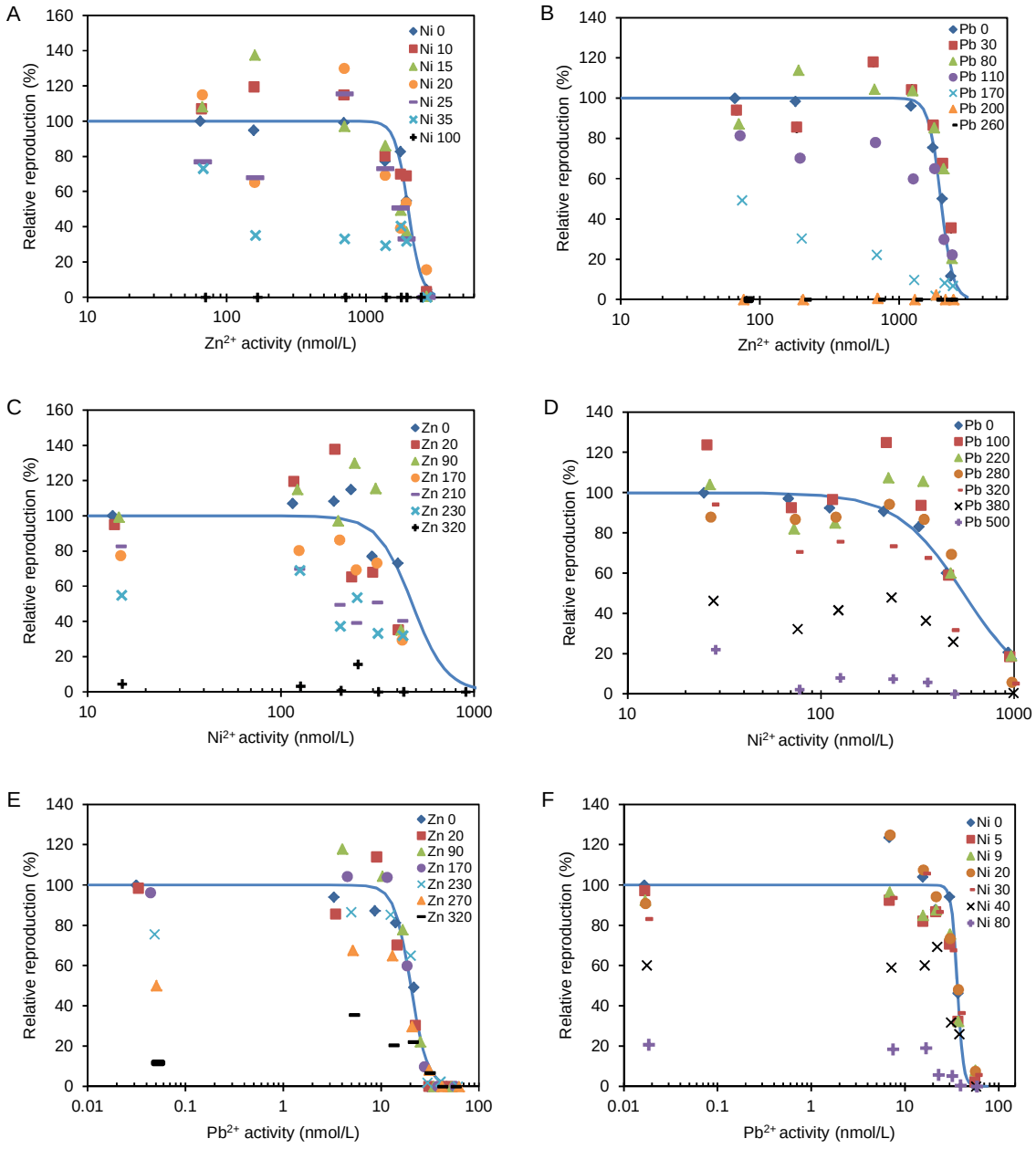

Figure 6.1. Dose- response data for 7d-reproduction relative to the control (no metals added; symbols) of *Ceriodaphnia dubia* as a function of free ion activity in binary mixtures of Ni, Zn, and Pb. A & C are data of Exp. 1 (Ni-Zn mixture), B& E of Exp. 2 (Pb-Zn mixture) and D & F of Exp. 3 (Ni-Pb mixture). Lines represent the fitted log-logistic dose-response curves of the individual metal exposures (Equation 6.4, lines) Parameters of the dose-response curves are reported in Table 6.2. Standard errors are reported in Table C.3-5 in Appendix C.

Table 6.1 Main physicochemical characteristics of the test waters used for investigation of 7d-reproductive toxicity of Ni, Zn, and Pb mixtures to *Ceriodaphnia dubia*.

Test id	pH	DOC (mg/L)	Na (mg/L)	Mg (mg/L)	K (mg/L)	Ca (mg/L)	SO4 (mg/L)	Cl (mg/L)	DIC (mg/L)
Exp. 1: Ni-Zn	7.1±0.2[a]	3.5±0.7	8.0±0.1	3.8±0.1	2.1±0.3	33±2	9.6±0.2	68±2	3.7±0.1
Exp. 2: Pb-Zn	7.1±0.1	3.9±0.8	6.9±0.2	3.7±0.1	2.0±0.2	32±1	9.6±0.2	68±0	1.9±0.1
Exp. 3: Ni-Pb & Ni-Pb-Zn	7.4±0.4	5.9±0.3	7.8±0.2	3.8±0.1	3.0±0.3	35±1	10±0	62±1	3.4±0.5

[a] Reproted values are arithmetic means of all measurements±standard deviation
DOC=dissolved organic carbon; DIC=dissolved inorganic carbon

Table 6.2. Parameters of the dose-response curves for the single Ni, Zn, and Pb exposures with *Ceriodaphnia dubia* based on filtered metal concentrations (Me_{diss}) and free metal ion activity (Me^{2+}) expressed based on 7d-reproductive toxicity.

		Dissolved concentrations (µg/L)			Free ion activities (nmol/L)		
Test id		Ni_{diss}	Zn_{diss}	Pb_{diss}	Ni^{2+}	Zn^{2+}	Pb^{2+}
Exp. 1: Ni-Zn	EC50	43±9	234±10	-	478±104	2014±106	-
	β	5.09±4.31	9.91±5.14	-	4.91±4.62	9.16±4.78	-
Exp. 2: Pb-Zn	EC50	-	239±4	111±6	-	1993±74	20±1
	β	-	8.59±3.32	6.87±1.94	-	9.93.±3.78	5.16±1.43
Exp. 3: Ni-Pb & Ni-Pb-Zn	EC50	52±2	197±17	302±8	546±70	1290±132	36±1
	β	2.75±0.78	3.62±1.20	20.46±8.00	2.52±0.71	3.16±1.11	14.0±8.9

EC50=50% effective concentration
B=slope parameter

6.3.1 Mixture effect analysis of Ni, Zn, and Pb

For all binary metal mixture treatments, the CA- and IA-predicted RR and the observed RR are shown as a function of the sum of TU_{Me2+} in Figure 6.2 and as a function of the sum of TU_{Mediss} in Figure C.4. While predictions with the IA model were relatively close to the observations for all three binary mixture experiments, the predicted reproduction of the CA model was mostly lower than the observed reproduction (Figure 6.2, Figure 6.3, Figure C.4). This suggests a trend towards antagonistic interactions relative to the CA model, which was confirmed by the statistical analysis of interactive mixture effects. For all binary mixture combinations the deviation parameter *a* of the CA model was positive both when the analysis was based on dissolved concentrations or on free ion activities, indicating significant antagonistic interactions (in all cases $p<0.001$; Table C.6-8; Figure C.5-6). No significant mixture interactions were observed relative to the IA model for the binary metal combinations, either expressed on dissolved metal concentrations or free ion activities ($p>0.05$), except for a significant antagonistic interaction relative to the IA model in the Pb-Zn mixture when expressed as free ion activities ($p=0.01$; Table C.6-8, Figure C5-6).

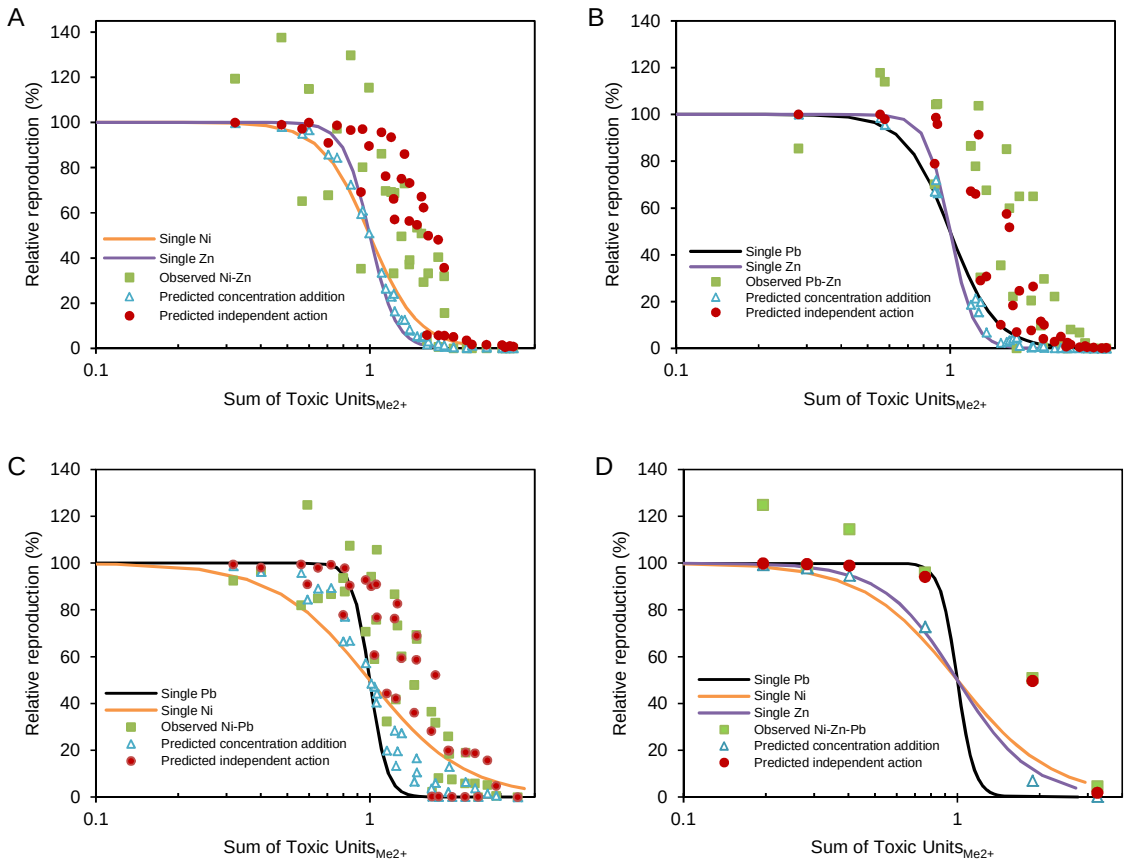

Figure 6.2. Observed and predicted reproduction relative to the control (no metals added) of *Ceriodaphnia dubia* in the binary mixture combinations of Ni, Zn, and Pb as a function of the sum of toxic units based on free ion activities for the Ni-Zn mixture (Exp. 1; A), Pb-Zn mixture (Exp. 2; B), the Ni-Pb mixture (Exp. 3; C), and the Ni-Pb-Zn-mixture (Exp. 3; D). Symbols are denoted as follows: observed reproduction (squares), predictions of concentration addition model (Equation 6.5, triangles), and predictions of independent action model (Equation 6.6, circles). Predictions are based on the parameters ($EC50_{Me2+,i}$ and $\beta_{Me2+,i}$) of the individual dose-response curves of Ni, Zn, and Pb (Table 6.2, lines). Lines represent dose-response curves of the individual Ni, Zn, and Pb exposures.

The predictions of daphnid reproduction with the CA and the IA reference models of the ternary mixture treatments and the observations are plotted as a function of sum of TU_{Me2+} and TU_{Mediss} in Figure 6.2.D and Figure C.4D, respectively. The IA predictions were relatively close to the observations. At low sum of toxic units, the predictions of the CA model were also relatively close to the observed values, but at higher toxic units ($\Sigma TU>0.7$) CA predicted reproduction was considerably lower than that observed. The statistical analysis of the interactive mixture effects showed that the ternary Ni- Zn-Pb mixture acted antagonistically relative to CA model, but non-

interactively relative to the IA model both for the dissolved concentrations and the free ion activities (Figure C.7; Table C.9).

For all mixture combinations, the AIC (Akaike Information Criterion), which is a measure of the model fit, was lower for the IA model (i.e. better fit) compared to the CA model (Table C.6-9).

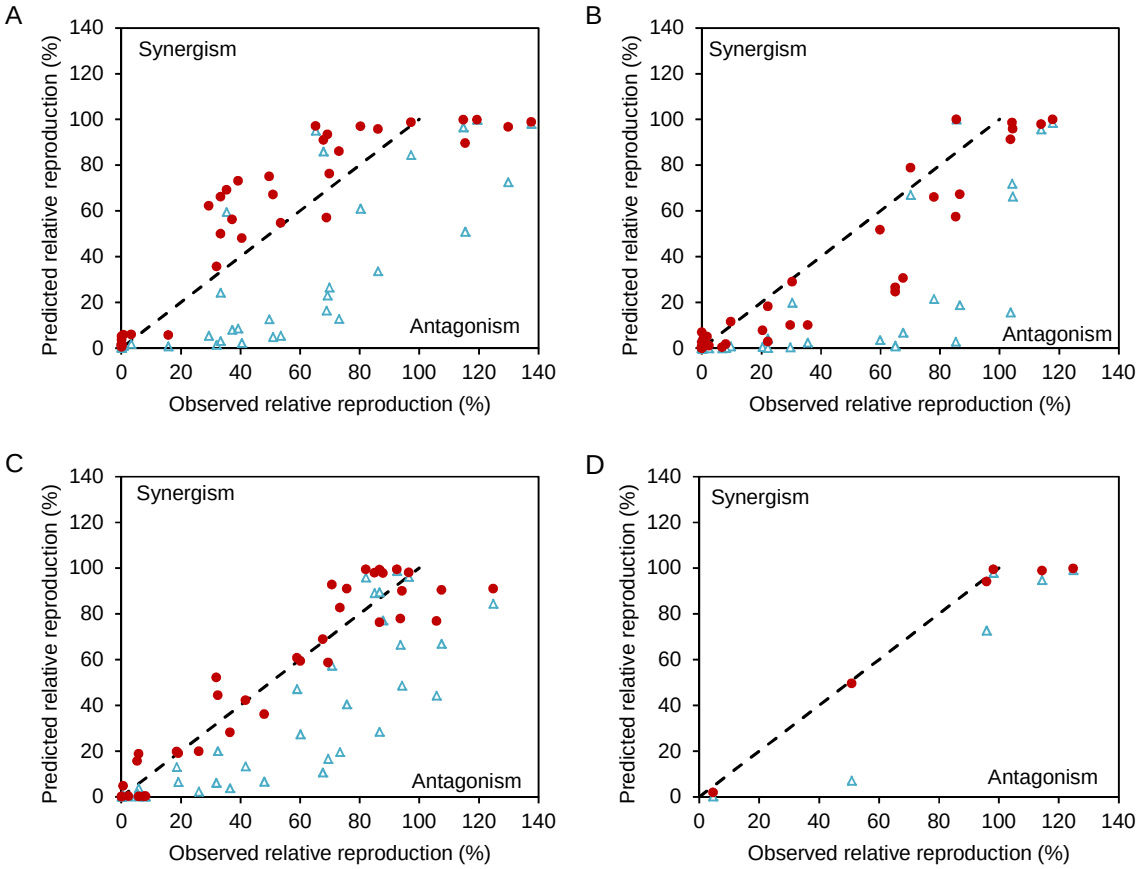

Figure 6.3. Observed 7d-relative reproductions versus predicted relative reproduction for the mixture reference models concentration addition (triangles) and independent action (circles) during the exposure of *Ceriodaphnia dubia* to the binary Ni-Zn (Exp. 1; A), Pb-Zn (Exp. 2; B), Ni-Pb (Exp. 3; C) and Ni-Pb-Zn (Exp. 4; D) mixture. Predictions are based on the parameters ($EC50_{Me2+,i}$ and $\beta_{Me2+,i}$) of single metal dose-responses of Ni, Zn, and Pb (Table 2).

6.4 Discussion

In the present study, we investigated the interactive effects of all possible binary and ternary mixture combinations of Ni, Zn, and Pb on *Ceriodapnia dubia* reproduction. The type of interactive effect observed in the metal mixtures was dependent on the reference model used

for analysing the interactive mixture effects. Ni, Zn, and Pb acted antagonistically relative to the concentration addition (CA) model in all binary and ternary mixture combinations tested. Mainly non-interactive mixture effects were observed relative to the independent action (IA) model.

Our results support the findings of Cooper et al. (2009) that the binary Pb-Zn mixture has an antagonistic effect on reproductive toxicity to *C. dubia* relative to the CA model (analysis based on total concentrations). In contrast with the present study, binary Ni-Zn mixtures acted synergistically relative to the IA model and exhibited non-interaction relative to the CA model for *Daphnia magna* reproduction (Chapter 5). This may suggest that the joint effect of Ni-Zn mixtures on reproduction are different between *D. magna* and *C. dubia*. Shaw et al. (2006) suggested that metal mixture interactive effects observed for *D. magna* may be different for different members of the same family (Daphniidae). Indeed, while the binary Cd-Zn mixture acted antagonistically on acute toxicity to three different daphnid species, including *C. dubia*, it acted in a non-interactive manner to *D. magna,* relative to the IA model (Shaw et al. 2006). The type of interactive effect observed in mixtures has been related to the steepness of the dose-response curves of the individual mixture components (Broderius et al. 1995; Chen & Lu 2002). Mixtures of components with steep dose-response curves often show antagonistic interactions according to the CA models, while less steep slopes more often result in strict non-interactivity (Shaw et al. 2006; Broderius et al. 1995) or synergistic interactions (Chen & Lu 2002). This observation may be a consequence of mathematical analysis of the mixture effects: steep slopes of single contaminant dose-response curves suggest that a cocktail of low doses yielding small effects individually causes large mixture effects when assuming an identical mode of action (CA) while no such effects may be noted if the components do not have an identical mode of action (IA) (Kortenkamp & Altenburger 2011). We observed that the slopes of the dose-response curves for Ni and Zn were in general steeper for *C. dubia* (present study: range of $\beta_{Ni2+ \text{ or } Zn2+}$=3-9) than for *D. magna* (Chapter 5: range of $\beta_{Ni2+ \text{ or } Zn2+}$=2-6), suggesting that the observed difference in type of interactive effects between *D. magna* and *C. dubia* may be attributed to differences in slope of the dose-response curve. (Figure 6.4) Alternatively, it is also possible that the difference in type of interactive effect between *C. dubia* and *D. magna* is partly attributable to differences in water chemistry between both studies, since the type of metal mixture interactive effect observed may be dependent on water chemistry (e.g. Versieren et al. 2014). The possible differences in type of interactive mixture effects between *C. dubia* and *D. magna* warns against

the extrapolation of interactive effects between species, even when they are closely related. Investigations into the mechanistic basis of these mixture interactions (e.g. at the bioaccumulation level) may lead to a better understanding of differences in observed interactive mixture effects between species.

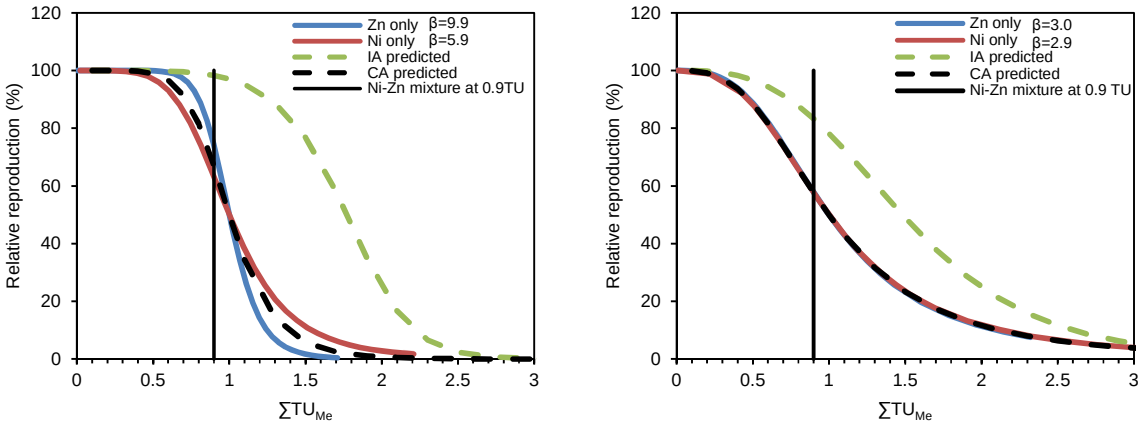

Figure 6.4. Predicted relative reproduction according to the independent action (IA, green dashed line) and to the concentration addition (CA, black dashed line) model for an equitoxic Ni-Zn mixture with *Ceriodaphnia dubia* (left panel, β_{Zn}=9.9 and β_{Ni}=5.9) and *Daphnia magna* (right panel, β_{Zn}=3.0 and β_{Ni}=2.9). Slopes and EC50 for Ni & Zn toxicity to *C. dubia* were taken from Table 6.2 (Exp. 1: Ni-Zn) and to *D. magna* from Table 5.1 (Natural DOC test series. Steep slopes of single contaminant dose-response curves such as for *C. dubia* suggest that a cocktail of low doses yielding small effects individually causes large mixture effects when assuming an identical mode of action (CA) while no such effects may be noted if the components do not have an identical mode of action (IA), e.g. for the example of an equitoxic Ni-Zn mixture at 0.9 TU (vertical black line) CA predicted effects are relatively large (~35% effect), while according to the IA model no effects would occur. The IA model for less steep slopes of single contaminant dose-response curves at the same mixture concentration will already predict some mixture effect & the difference between IA & CA will be smaller, often resulting in non-interactive mixture effects relative to the CA model.

As far as we are aware, no other studies have investigated the interactive effects of binary Ni-Pb or ternary Ni-Zn-Pb mixtures at the life-history level for invertebrates. However, our observation of CA antagonistic effects and IA non-interactive effects for all binary and ternary mixture combinations of Ni, Zn, and Pb are in line with interactive effects observed at the level of metal uptake. In a multi-metal experiment, uptake of Pb by *D. magna* was not affected in the presence of Ni and Zn and also Pb did not influence the uptake of Ni and Zn. Nickel and Zn on the other hand did have a competitive effect (antagonism) on each other's uptake

(Komjarova & Blust 2008). When *D. magna* was exposed to a mixture of Ni and Pb different mRNA transcription patterns were observed compared to when it was only exposed to the single components (Vandenbrouck et al. 2009), which suggests that the Ni-Pb mixture acts interactively at the sub-organismal level.

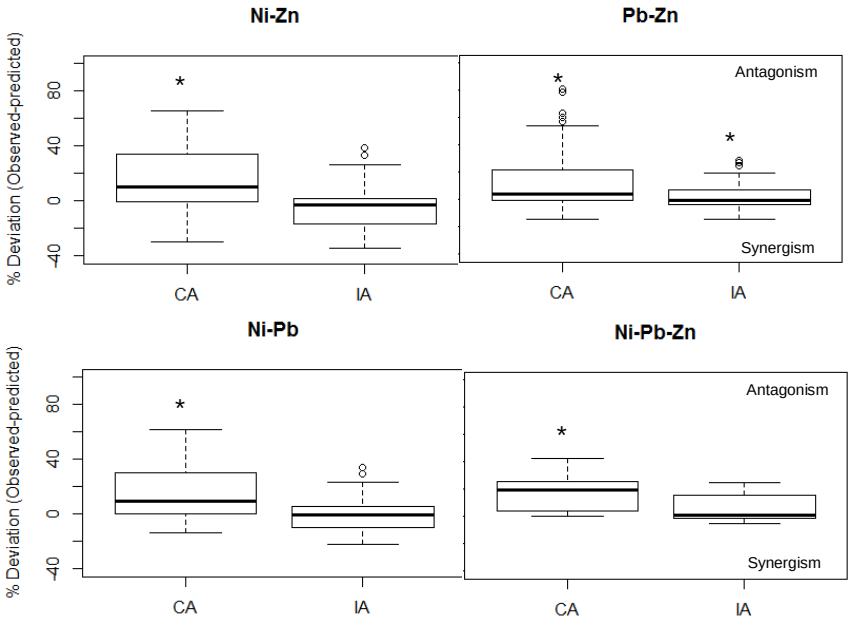

Figure 6.5. Deviation (%) between observed 7d-relative reproduction and concentration addition (CA) and independent action (IA) predicted relative reproduction (%) for *Ceriodaphnia dubia* for the different mixtures. Predictions were based on the free ion activity parameters ($EC50_{Me2+,i}$ and $\beta_{Me2+,i}$) of the individual dose-response curves of Ni, Zn, and Pb (Table 6.2). Median values are given in bold, bottom and top of the boxplots give the 25th and 75th percentile. Bottom and top of whiskers represent the 5th and 95th percentile. Circles are outliers. Positive values indicate a trend towards antagonistic deviations, negative values towards synergistic deviation. Asterisks indicate if deviations were significantly different from non-interactivity.

The IA model generally fitted the observed toxicity of binary and ternary mixture combinations of Ni, Zn, and Pb better than the CA model (Figure 6.5 and Figure C.8). This observation confirms our expectations based on the different modes of action of Ni, Zn, and Pb. Indeed, chronic Zn toxicity to daphnids has been linked to the disturbance of Ca homeostasis (Muyssen et al. 2006), while chronic Ni exposure affected the Mg homeostasis (Pane et al. 2003). For fish Pb toxicity has also been linked to disruption of Ca homeostasis (Rogers & Wood 2004). However, disturbance of the Ca homeostasis does not appear to be the primary mechanism of Pb toxicity to invertebrates (Mager et al. 2011a; Brix et al. 2012; Chapter 2) and the main mode

of action remains thus unclear. These differences in modes of action between the different metals are also reflected in their respective chronic daphnid bioavailability models (Heijerick et al. 2005; De Schamphelaere et al. 2006; Deleebeeck et al. 2008; Chapter 2 & 4), which are dissimilar both in structure and in the competitive cations considered (visualized in Appendix C: Figure C.9).

Several studies have investigated the validity of the IA model for predicting mixture toxicity of substances with different modes of action (e.g. Backhaus et al. 2000; Faust et al. 2003; Cedergreen et al. 2008). Some studies found that the IA model predicted toxicity of mixtures of dissimilar acting substances clearly better than the CA model (Backhaus et al. 2000; Faust et al. 2003), although this observation was not supported by Cedergreen et al. (2008). The differences in predictions of joint toxicity between the CA and IA model are often small (Backhaus et al. 2000). Given that the CA reference model is generally the most conservative model (e.g., Cedergreen et al. 2008), this model type has been proposed for use in a first tier in risk assessment approaches for mixtures (Backhaus & Faust 2012). In the present study, the CA model was also the most conservative model. However, it should be noted that the CA approach will result in an overestimation of the effects of Ni, Zn, and Pb on daphnid reproductive toxicity. Based on this study, both the CA model as well as the IA model provided conservative predictions for Ni, Zn, and Pb mixture toxicity, since no significant synergistic interactions were observed for any of the mixtures. Therefore, an IA-based approach appears to be, at least for *C. dubia*, a more appropriate approach to evaluate effects for Ni, Zn, and Pb mixtures.

The type of interactive effect observed may change depending on how exposure is expressed (e.g. dissolved concentrations vs. free ion activities) (e.g. Meyer et al. 2015b). In the present study, the type of interactive effect observed generally did not differ when the statistical analysis were based on dissolved concentrations or on calculated free ion activities. The only exception was the binary Pb-Zn mixture, for which, relative to IA, non-interactive effects were found based on dissolved concentrations and antagonistic effects based on free ion activities (Table C.7). This shift in type of interactive effect can be attributed to chemical speciation effects. In mixtures Pb, Ni, and Zn will compete for DOC binding sites, resulting in less Pb, Ni, and Zn bound to the DOC in mixture treatments than in the corresponding individual metal treatments. This results in more free Pb^{2+}, Ni^{2+} and Zn^{2+} in the mixture solution compared to the

respective individual Pb, Ni and Zn treatments (Figure C.10). Because the fractions of free ion in the mixture treatments are higher than in the individual metal treatments, the relative reproduction predicted with the IA model based on free metal activities is consistently lower than the predictions based on dissolved concentrations (all points below the 1:1 reference line in Figure C.11). This difference is most prominent for the binary Pb-Zn mixture (Figure C.11B). Indeed, the statistical mixture analysis showed a significant antagonistic interaction based on the free metal concentrations, while the effects were statistically non-interactive based on dissolved concentrations. These effects were less obvious in the binary Ni-Zn and Ni-Pb mixtures, since WHAM VII predicts that Ni binds less to DOC than Pb and Zn (Figure C.12).

Given the influence of chemical speciation effects on interactive mixture effects, it can be concluded that it is important to analyse metal mixture interactions both based on dissolved concentrations as on measured or calculated metal activities. Due to similar geochemical effects, combined mixture effects can be judged synergistic, which are of main concern in environmental risk assessments, when analysed at the dissolved level, but can be truly non-interactive effects at the actual bioavailable level (free ion activities), (e.g. Meyer et al. 2015b).

Despite the considerable research available, it is clear that we do not yet fully understand how metals interact in mixtures. This is partly due to the fact that mechanistic studies are still largely lacking (but see e.g. Komjarova & Blust 2008; Norwood et al. 2007; 2013). This currently hampers the development of models to predict metal mixture toxicity and the incorporation of metal mixture toxicity in risk assessment frameworks (Meyer et al. 2015). In the present study, the observed mixture toxicity of binary and ternary combinations of Ni, Zn, and Pb on *C. dubia* reproduction could be explained based on the IA theory, thereby assuming different modes of action. However, to be able to integrate mixture toxicity in metal risk assessment frameworks, the focus in metal mixture studies should shift from solely investigating interactive effects, towards building models that can accurately predict metal mixture toxicity. These models should ideally take into account geochemical effects as well as possible competitive effects and other interactions at biological surfaces, which are crucial in describing metal mixture toxicity. Risk assessments of individual metals now use chronic bioavailability models that account for effects of water chemistry on metal bioavailability and toxicity, e.g. the biotic ligand model (BLM) (e.g. DEPA 2008; Van Sprang et al. 2009). The BLM-concept has also recently been proven successful in predicting the acute toxicity of metal mixtures (e.g. Versieren et al. 2014; Iwasaki et al. 2015;

Santore & Ryan 2015). Additionally, also the WHAM-F_{TOX}, an alternative metal mixture bioavailability model which uses metal binding to HA as a surrogate to model competition and metal binding at the cell surface of aquatic organisms, has been shown to accurately predict metal mixture toxicity to aquatic organisms (Tipping & Lofts 2013; 2015). In an evaluation of four metal mixture bioavailability models using different assumptions, it was observed that models based on the IA approach generally give better predictions than those based on the CA approach (Farley et al. 2015; Van Genderen et al. 2015), corroborating the overall conclusions of our present study. However, the application of these models to chronic mixture toxicity datasets remains to be further tested (but see Santore & Ryan).

7

Validation of a metal mixture bioavailability model combining the individual metal biotic ligand models with the independent action model to predict chronic Zn-Ni-Pb mixture toxicity to *Ceriodaphnia dubia*

7. Validation of a metal mixture bioavailability model combining the individual metal biotic ligand models with the independent action model to predict chronic Zn-Ni-Pb mixture toxicity to *Ceriodaphnia dubia*

7.1 Introduction

Although metals in the aquatic environment mostly occur as mixtures, risk assessment procedures currently consider only single metal toxicity. Metal mixture effects have been reported to be very variable. Both non-interactivity, antagonism, as well as synergism have been observed in acute metal mixture studies, depending on the test organisms, metal combination, metal concentration ratios, metal concentrations, and considered endpoints (Norwood et al. 2003; Vijver et al. 2011).

To integrate metal mixture toxicity in risk assessment frameworks, models that can accurately predict metal mixture toxicity are needed (Meyer et al. 2015, Van Genderen et al. 2015). The incorporation of the effects of water chemistry in these metal mixture models is crucial, since the physico-chemical composition of the receiving water determines the bioavailability and thus the toxicity of metals to aquatic organism (e.g. Heijerick et al. 2005; Deleebeeck et al. 2008; Chapter 2). In the past decade, several bioavailability models have been developed that are able to predict acute (e.g. Di Toro et al. 2002; De Schamphelaere & Janssen 2002) and chronic toxicity of metals (e.g. Heijerick et al. 2005; Deleebeeck et al. 2008; Chapter 2) in the aquatic environment. These models typically relate metal toxicity to the concentration of free metal ion binding at the biotic ligand (BL), i.e. a cell surface receptor, and the activity of certain cations (e.g., $Ca^{2+,}$ H^{+} , Mg^{2+}), which compete with the metal for binding at the BL (Di Toro et al. 2001). Bioavailability models for individual metals are currently increasingly being incorporated in risk assessment procedures for individual metals in Europe (Van Sprang et al. 2009; 2016) and the United States (USEPA, 2007). Recently, several bioavailability based metal mixture models have been shown to be successful in predicting the acute metal mixture toxicity to a diverse range of (aquatic) organisms (e.g. Versieren et al. 2014; Iwasaki et al. 2015; Santore and Ryan 2015; Tipping and Lofts 2015), but the application of these models to chronic data remains largely to be tested (Meyer et al. 2015; Van Genderen et al. 2015; but see Santore and Ryan, 2015). Therefore, we investigated whether chronic metal mixture toxicity to *Ceriodaphnia dubia* can be predicted using a chronic metal mixture bioavailability model.

The actual objectives of the present study were threefold. First, we wanted to investigate if interactive effects shifted depending whether the metals (i.e. Ni, Zn, and Pb) were combined in equitoxic concentrations or in environmentally realistic metal concentration ratios. Often metal mixture toxicity is investigated at equitoxic concentrations. However, the metal concentration ratios in equitoxic designs may not be representative for environmental realistic exposure scenarios. Since the type of interactive effect of a mixture may depend on the applied metal concentration ratio (Norwood et al. 2003) conclusions on the type of interactive effects based on equitoxic experiments might not be applicable for environmental relevant metal concentration ratios.

The second objective was to investigate if the interactive effects in Ni-Zn-Pb mixtures are influenced by varying water chemistry. Previously, it was observed that Ni-Zn-Pb mixtures acted antagonistically on *C. dubia* reproduction relative to the concentration addition (CA) model (evaluated in a single medium), while it was non-interactive relative to the independent action (IA) model (Chapter 6). However, it has been reported that shifts in interactive effects can occur due to varying physico-chemistry (e.g. Norwood et al. 2003, Versieren et al. 2014), because water chemistry variables, such as hardness ions and H^+, may influence the competitive interactions between metals at the biotic ligand sites.

The third objective was to investigate whether chronic toxicity of Ni-Zn-Pb mixtures to *C. dubia* can be predicted using a chronic metal mixture bioavailability model (MMBM). Based on the results in Chapter 6, we developed a MMBM that combines the individual bioavailability models for Ni (De Schamphelaere et al. 2006), Pb (Chapter 2) and Zn (Chapter 4) with the IA model. We hypothesised that metal mixture toxicity can be predicted using a MMBM that is calibrated on the toxicity data of the individual metals.

To address these study objectives, we investigated the reproductive toxicity of the ternary Ni-Zn-Pb mixtures in three separate experiments using (modified) natural water. Each experiment consisted of two test media differing in physico-chemical properties. In one experiment, the individual effect of pH on chronic mixture toxicity was investigated, while in a second experiment the individual effect of Ca was evaluated. The third experiment consisted of two natural waters differing in water chemistry (e.g., pH, dissolved organic carbon (DOC), Ca,...). For every test

medium, the interactive effects were investigated in two mixture rays differing in the metal concentration ratio, i.e. an '*equitoxic*' ray and an '*environmental*' ray.

7.2 Methods

7.2.1 Test design and preparation of test media

All toxicity tests were conducted in (modified) natural water. The natural water was sampled from the stream L'Ourthe Orientale (Brisy, Belgium) and from the Ankeveensche Plassen lake system (Ankeveen, the Netherlands). These waters have previously been used for metal toxicity testing with daphnids in our laboratory (e.g., De Schamphelaere et al. 2005, Deleebeeck et al. 2008, Chapter 2). Water was filtered (0.2 µm) on site and collected in acid-washed poly-ethylene barrels. Upon arrival in the lab, the water was stored in total darkness at 4°C until further use.

The effects of water chemistry on chronic toxicity of Zn-Ni-Pb mixtures were investigated in three separate experiments. In each experiment, toxicity was investigated in two different test media, either differing in pH, Ca or natural water. In all experiments, the basic medium was the Brisy base medium (natural Brisy water +0.5 mM Ca and pH 7), relative to which potential shifts in interactive effects due to different water chemistry were investigated. The inclusion of this Brisy base medium in all three experiments as one of two test waters allowed us to account for inter-test variability of mixture effects (i.e. if mixture effects would not be reproducible between experiments). 0.5 mM Ca (using $CaCl_2$) was added in the Brisy base medium as the low Ca concentrations in the Brisy water might induce physiological Ca stress to *C. dubia* (Chapter 2).

The pH experiment consisted of the "Brisy base" medium and "Brisy pH 8" medium (Brisy water +0.5 mM Ca added and pH 8). The Ca experiment consisted of the "Brisy base" medium and "Brisy Ca 2.0 mM" medium (Brisy water + 1.75 mM Ca and pH 7). The natural water experiment consisted of the "Brisy base" medium and the "Ankeveen" medium. The water chemistry of all test waters is reported in Table 7.1.

Table 7.1. Main physicochemical characteristics[a] of the solutions used for reproductive toxicity tesing of Ni-Zn-Pb mixtures with *Ceriodaphnia dubia*.

Experiment	Test water ID	pH	DOC (mg/L)	Ca (mg/L)	Mg (mg/L)	Na (mg/L)	K (mg/L)	Cl[b] (mg/L)	SO_4[b] (mg/L)	DIC (mg/L)
pH	Brisy pH base	7.1±0.1	5.2±0.9	32±0	4.3±0.0	58±1	2.3±0.1	56	114	4.2±0.1
	Brisy pH 8	8.1±0.1	5.4±0.8	33±1	4.4±0.2	59±2	2.6±0.4	54	7.7	27±0
Ca	Brisy Ca base	7.1±0.6	6.1±1.3	33±1	4.5±0.2	55±2	2.6±0.3	56	112	3.5±0.0
	Brisy Ca 2 mM	7.2±0.1	5.9±1.2	75±1	4.4±0.1	55±1	2.4±0.2	174	114	3.3±0.1
Natural waters	Brisy nat base	7.2±0.4	5.1±0.7	33±1	4.5±0.1	59±1	2.5±0.3	54	113	3.9±0.4
	Ankeveen	8.0±0.6	11.2±2.2	45±1	12±0	63±1	7.5±0.3	115	60	18±0

[a] Mean measured concentrations ± standard deviation are reported
[b] Cl and SO_4 were measured on a mixed sample of all test concentration for each individual water, therefore no standard deviation is available.
DOC= Dissolved organic carbon; DIC= dissolved inorganic carbon

The Ni-Zn-Pb mixture toxicity was investigated using a ray design (see Introduction). Two mixture rays were tested for every test medium: an *equitoxic ray* and an *environmental ray*. In the *equitoxic ray*, mixture toxicity was investigated at equitoxic concentrations based on their toxic units (TU). The TU was defined relative to the median effective concentration (EC50) using Equation 7.1.

$$TU_i = \frac{c_i}{EC50_i} \quad (7.1)$$

In Equation 7.1, TU_i is the TU of metal *i*, c_i is the dissolved concentration of metal *I* (µg/L), and $EC50_i$ is the median effective concentration of metal *I*, expressed as dissolved concentration (µg/L). $EC50_i$ in the different test waters were predicted using the chronic *C. dubia* bioavailability models for Ni (De Schamphelaere et al. 2006), Zn (Chapter 4) and Pb (Chapter 2). The nominal sum of TU (∑TU) range was 0.1-3 $\sum TU_{Mediss}$.

In the *environmental ray*, toxicity of Ni-Zn-Pb mixtures was investigated at the median Ni-Zn-Pb concentration ratio occurring in the Dommel, a tributary of the Meuse in the Netherlands. Dissolved Ni and Zn concentrations in this river system have been shown to be higher than their individual HC5 values, and thus ecosystems may be potentially affected by metal contamination (Verschoor et al. 2011). Metal concentration ratios were calculated based on dissolved Ni, Zn and Pb concentrations, expressed as µg/L, measured in 405 samples in over 80 sampling sites in the Dommel river basin in 2010. The median Zn:Ni concentration ratio in these samples was 3.0 (5^{th}-95^{th} % percentile: 0.6-33; Figure 7.1). The median Zn:Pb concentration ratio was 179 (5^{th}-95^{th} % percentile: 22-1142). Each mixture ray was investigated at 6 mixture concentrations. Additionally, for each test medium, the concentration response of Ni, Zn, and Pb individually

were investigated using 5 concentration treatments per metal. Finally, for each test medium a control (i.e. no Ni, Zn or Pb added) was also investigated. All treatments within an experiment (control + indiv idual metal treatments + mixture treatments for both test waters) were tested simultaneously, to avoid possible interference of temporal sensitivity shifts in later data interpretation (De Laender et al. 2009).

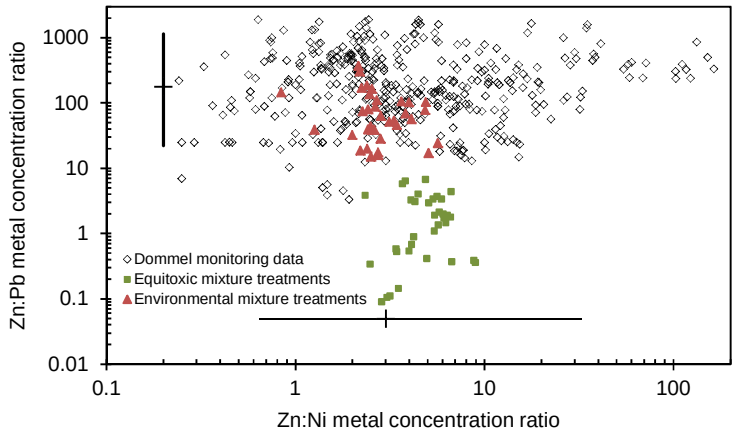

Figure 7.1. Metal mixture concentration ratio plot: Zn:Ni metal concentration ratio as a function of the Zn:Pb metal concentration in the solution. Metal concentration ratios were calculated based on dissolved metal concentrations (expressed as µg/L). Symbols are denoted as follows: diamonds are metal ratios in the Dommel monitoring dataset, squares denote metal ratios in the *equitoxic* mixture treatments, and triangles are metal ratios in the *environmental* mixture treatments. Lines denote the 5th-95th percentile of the Zn:Ni (horizontal) and Zn: Pb (vertical) concentration ratios in the Dommel monitoring dataset. Crosses are the median of metal concentration ratios in the Dommel monitoring dataset.

7.2.2 Ecotoxicity testing with *C. dubia*

Reproductive toxicity assays with *C. dubia* were conducted following the US Environmental Protection protocol (USEPA 2002a). In short, juveniles originated from an in-house isoclonal lab culture, which has been maintained for more than 20 years at 25°C in activated carbon-filtered Ghent city tapwater to which vitamins (75 µg/L thiamine, 1µg/L cyanocobalamin, and 0.75 µg/L biotin) and selenium (1 µg Se/L) are added. Daphnids were individually acclimated to the test media for one generation (1 week) in poly-ethylene cups containing 15 mL of control test medium (1 daphnid per cup). Media were completely renewed three times during the acclimation period. During acclimation and testing periods, daphnids were kept at 25°C under a 16h:8h

light:dark cycle and they were daily fed with *Pseudokirchneriella subcapitata* ($2x10^5$ cells/mL) and a Yeast-Urtica-Trout Chow mixture (12 mg solids/L). Tests were initiated with juveniles originating from the third brood, and only from mothers that produced at least 20 juveniles in total during their first three broods and that produced at least 8 juveniles in the third brood (USEPA, 2002a). Tests were conducted in polyethylene cups containing 15 mL of the test medium. Juveniles (<24h old, 1 per replicate) were distributed among all treatments as prescribed by the USEPA protocol (2002a). Test media were completely renewed daily. Before renewal, fresh test media were adjusted to the required pH by adding dilute HCl or NaOH. Parent mortality and number of juveniles were scored daily. The total number of juveniles is further called "total reproduction" or "R". The toxicity tests were ended when at least 60% of the control animals had produced three broods (USEPA 2002a). Test validity was evaluated relative to the criteria prescribed by the USEPA protocol (2002a), i.e. control mortality should be less than 20% and control organisms should produce on average at least 15 juveniles.

7.2.3 Analytical chemistry

During the test period, samples for total and dissolved (filtered through 0.45 µM Acrodisc, PALL Life Science) metal analysis in fresh medium (new medium just before transfer of daphnids to the cups) were taken from all treatments on days 0 and 6. Samples for metal analysis in dissolved old medium (sample of medium just after transfer of daphnids to a new cup) were taken from all treatments on days 1 and 7. Samples for metal analysis were acidified to 0.14 mol/L HNO_3. Concentrations of total and dissolved Zn (all concentrations), Ni (concentrations >5 µg/L) and Pb (concentrations >10 µg/L) were measured using inductive coupled plasma-optical emission spectroscopy (ICP-OES, iCAP 7200 dual, Thermo Scientific; Reference Material TM-28.4 [Environment Canada]). Concentrations of total and dissolved Ni (concentrations <5 µg/L) and Pb (concentrations <10 µg/L) were measured using graphite furnace atomic absorption spectrophotometry (GFAAS Furnace Autosampler; Thermo Fisher Scientific; Reference Material TM-24.3, lot 0510 [Environment Canada]: limit of quantification 1 µg Ni/L and 1.4 µg Pb/L, method detection limit 0.3 µg Ni/L and 0.4 µg Pb/L). Dissolved concentrations of major cations (Ca, Mg, Na, K) were measured on a subset of the samples used for Ni, Zn, and Pb analysis using ICP-OES (Reference Material Cranberry 05 [Environment Canada]). DOC and Disolved Inorganic Carbon (filtered through 0.45 µM Acrodisc, PALL Life Science) for randomly selected treatments

were measured with a Total Organic Carbon analyser (TOC-5000, Shimadzu). Cl and SO_4 samples were measured colormetrically (Aquamate, Thermo Electron Corporation; Chloride: Merck, Spectroquant 1.14897.001; Sulphate: Merck, Spectroquant 1.14548.001). The pH of fresh and old media were measured daily with a glass electrode (Hanna Instruments).

7.2.4 Chemical speciation calculations

Chemical speciation was calculated with the Windermere Humic Aqueous Model VII (WHAM VII) (Tipping et al. 2011). We assumed that dissolved organic matter (DOM) contains 50% carbon on a weight basis (Ritchie & Perdue 2003). Furthermore, we assumed that 65% of the DOM consisted of active fulvic acid (FA), with the other fraction being inert for metal binding. Consequently, the measured DOC concentration was multiplied by 1.3 to calculate the FA concentration used in the input for the speciation calculations. Additionally, the competing effects of Fe^{3+} for DOC binding sites were considered by assuming that Fe^{3+} activity is controlled by $Fe(OH)_3$ using the default equation and solubility product embedded in WHAM VII (Loft & Tipping 2011). Similar assumptions have been used to model metal-FA complexation in several recent metal mixture studies (e.g. Tipping & Lofts 2013; 2015; Iwasaki & Brinkman 2015). Finally, the default stability constants for complexation of Zn to inorganic ligands in WHAM VII were modified to those reported by the National Institute for Standards and Technology (Smith et al. 2004). These assumptions resulted in accurate predictions of individual chronic Ni, Zn, and Pb mixture toxicity to daphnids using their respective individual bioavailability models (Chapter 3 & 4).

7.2.5 Dose response analysis of single metal exposures

The dose response analysis was based on the relative reproduction as endpoint. The relative reproduction in treatment j of test medium k ($RR_{j,k}$; %) was defined as the total reproduction in treatment j of test medium k ($R_{j,k}$) relative to total control reproduction in test medium k ($R_{con,k}$) (Equation 7.2).

$$RR_{j,k} = \frac{R_{j,k}}{R_{con,k}} \times 100\% \quad (7.2)$$

Median, 20% and 10% effective concentrations (EC50, EC20 and EC10, respectively) and corresponding confidence intervals were determined for the individual exposures of Ni, Zn, and

Pb in all test media using the log-logistic dose response model with two parameters in Statistica 7 (StatSoft). The log-logistic dose response model fitting was based on average dissolved metal concentrations (calculated as the average of dissolved measured metal concentration in fresh and old media) as well as on the corresponding WHAM VII calculated free ion activities (Equation 7.3).

$$RR_{i,k} = \frac{100}{1+\left(\frac{c_{i,k}}{EC50_{i,k}}\right)^{\beta_{i,k}}} \quad (7.3)$$

In equation 7.3, $RR_{i,k}$ is the predicted relative reproduction (%) for metal *i* in test medium *k*, $c_{i,k}$ is the concentration of metal *i* in test medium *k*. $EC50_{i,k}$ is the median effective concentration of metal *i* in test medium *k* and $\beta_{i,k}$ is the slope parameter of metal *i* in test medium *k*.

7.2.6 Objective 1 & 2: Analysis of mixture interactions

The interactive effects in Ni-Zn-Pb mixtures were evaluated using the mixture analysis framework proposed by Jonker et al. (2005), following the methodology described by Hochmuth et al. (2014). This framework allows to evaluate if the observed relative reproduction in the mixture treatments deviates from the predicted relative reproduction, relative to the CA (Eq. 7.4) and IA (Eq. 7.5) models, both assuming strict non-interaction.

$$\sum_{i=1}^{n} \frac{c_{i,k}}{EC50_{i,k} \times \left(\frac{100-RR_{mix,CA}}{RR_{mix,CA}}\right)^{\frac{1}{\beta_{i,k}}}} = 1 \quad (7.4)$$

$$RR_{mix,IA} = 100 \times \prod_{i=1}^{n} \left(\frac{1}{1+\left(\frac{c_{i,k}}{EC50_{i,k}}\right)^{\beta_{i,k}}} \right) \quad (7.5)$$

Where $RR_{mix,CA}$ and $RR_{mix,IA}$ is the predicted RR (%) of the mixture following the CA and IA model, respectively. *n* is the number of components in the mixture.

The actual analysis was performed in three steps. In a first step, metal mixture toxicity (RR) was predicted based on the parameters of the single metal dose-response curves ($EC50_{i,k}$, *and* $\beta_{i,k}$ calculated with Equation 7.3) using either the CA (Eq. 7.4) or IA (Eq. 7.5) reference model. The predictions of this step are used to visualise mixture toxicity as a function of sum of toxic units (∑TU; Eq. 7.1) in Figure 7.4. The non-linear relationship of the CA model (Eq. 7.4) was solved iteratively for RR using the Solver function in Excel. In the second step,

Equation 7.4 and 7.5 were fitted to all data, both the single metal treatments and the mixture treatments. In step three, the reference models were extended with the deviation parameter *a*, which is a measure for the magnitude of potential interactive effects and thus tests for deviations of non-interactivity (Jonker et al. 2005; methodology described in Hochmuth et al. 2014). Step two and three were performed as described in Chapter 6 using the software R 3.1.10, by simultaneously taking 20 000 random parameter sets (i.e. $EC50_{Me,i}$ and $\beta_{Me,i}$) from a normal distribution with the mean and standard deviation originating from the single metal dose-response curves (calculated with Eq. 7.3; reported in Table 7.3). The mean relative reproduction of all treatments was used as input. To evaluate the effect of metal concentration ratio (objective 1) and water chemistry (objective 2) on the type of interactive effect observed, the analysis was performed for every mixture ray and every test water separately.

Since the type of interactive effects observed can shift depending on whether the mixture analysis is based on dissolved metal concentrations or free ion activities (e.g. Meyer et al. 2015b; Chapter 6), analyses were made for both expressions of exposure.

7.2.7 Objective 3: chronic metal mixture bioavailability model

For the sake of simplicity, we will further refer to all bioavailability models as BLMs. However, only the Pb model is an actual BLM, while the Ni & Zn models are bioavailability models. To integrate the individual chronic *C. dubia* BLMs into a metal mixture bioavailability model, it is crucial that these models are able to predict metal toxicity in the individual metal treatments. Therefore, we first evaluated the accuracy of these models to predict $EC50_{Mediss}$ in the test waters considered in the present study. More specifically, we used the chronic *C. dubia* Ni (De Schamphelaere et al. 2006; Eq. 3.3), Pb (Chapter 2; Eq. 2.4) and Zn (Chapter 4; Eq. 4.5) BLM to predict $EC50_{Nidiss}$, $EC50_{Pbdiss}$ and $EC50_{Zndiss}$ in the individual metal treatments, respectively. The calibrated intrinsic sensitivities ($Q50_{Ni2+}$, $Q50_{Zn2+}$ and $EC50^{*}_{Pb2+}$) of the MMBM reported in Table 7.2 were used (see further).

Since it was previously shown that Ni-Zn-Pb mixture toxicity was non-interactive relative to the IA model but antagonistic relative to the CA model (Chapter 6), the metal mixture toxicity to *C. dubia* was modeled in the MMBM based on the IA concept. The MMBM was developed by

combining the IA model (Eq. 7.5) with the individual chronic *C. dubia* biotic ligand models (BLM) for Ni (De Schamphelaere et al. 2006, Eq. 3.3), Zn (Chapter 4, Eq. 4.5) and Pb (Chapter 2, Eq. 2.4) in Equation 7.6.

$$RR_{MMBM,k} = 100 \times \frac{1}{1+\left(\frac{c_{Ni^{2+},k}}{EC50_{Ni^{2+},BLM,k}}\right)^{\beta_{Ni^{2+}}}} \times \frac{1}{1+\left(\frac{c_{Zn^{2+},k}}{EC50_{Zn^{2+},BLM,k}}\right)^{\beta_{Zn^{2+}}}} \times \frac{1}{1+\left(\frac{c_{Pb^{2+},k}}{EC50_{Pb^{2+},BLM,k}}\right)^{\beta_{Pb^{2+}}}} \quad (7.6)$$

In Equation 7.6, $RR_{MMBM,k}$ is the relative reproduction (%) in water *k* predicted with the MMBM. $c_{Ni2+,k}$, $c_{Zn2+,k}$ and $c_{Pb2+,k}$ is the WHAM VII predicted free metal activity of Ni^{2+}, Zn^{2+} and Pb^{2+} in test medium *k*, respectively. $EC50_{Ni2+,BLM,k}$, $EC50_{Zn2+,BLM,k}$ and $EC50_{Pb2+,BLM,k}$ are the 50% effective concentrations for Ni^{2+}, Zn^{2+} and Pb^{2+}, expressed as free metal activity, in solution *k* predicted using the chronic Ni (Eq. 3.3), Zn (Eq. 4.5) or Pb (Eq. 2.4) BLM, respectively. β_{Ni2+}, β_{Zn2+} and β_{Pb2+} are the slope parameters of the log-logistic dose response curves of Ni^{2+}, Zn^{2+} and Pb^{2+}. In the MMBM, it is assumed that the slopes are independent of the water chemistry. Substituting the chronic BLMs for Ni (De Schamphelaere et al. 2006, Eq. 3.3), Zn (Chapter 4, Eq. 4.5) and Pb (Chapter 2, Eq. 2.4) in Equation 7.6, gives the following full description of the chronic Ni-Zn-Pb MMBM for *C. dubia* (Eq. 7.7).

$$RR_{MMBM,k} =$$

$$100 \times \frac{1}{1+\left(\frac{c_{Ni^{2+},k}}{10^{-\left(Q50_{Ni^{2+}}+S_{pH,Ni}\times pH_k\right)}\left(1+K_{CaBL_{Ni}}\{Ca^{2+}\}_k+K_{MgBL_{Ni}}\{Mg^{2+}\}_k\right)}\right)^{\beta_{Ni^{2+}}}} \times$$

$$\frac{1}{1+\left(\frac{c_{Zn^{2+},k}}{10^{-\left(Q50_{Zn^{2+}}+S_{pH,}\times pH_k\right)}\left(1+K_{CaBL_{Zn}}\{Ca^{2+}\}_k+K_{MgBL_{Zn}}\{Mg^{2+}\}_k+K_{NaBL_{Zn}}\{Na^{+}\}_k\right)}\right)^{\beta_{Zn^{2+}}}} \times$$

$$\frac{1}{1+\left(\frac{c_{Pb^{2+},k}}{EC50^{*}_{Pb^{2+}}\left(1+K_{HBL_{Pb}}\{H^{+}\}_k\right)}\right)^{\beta_{Pb^{2+}}}} \quad (7.7)$$

In Equation 7.7, $Q50_{Ni2+}$ and $Q50_{Zn2+}$ are the intrinsic sensitivities of the chronic Ni and Zn BLM, respectively. $EC50^{*}_{Pb2+}$ is the intrinsic sensitivity of the Pb BLM. $S_{pH,Ni}$ and $S_{pH,Zn}$ is the pH slope of Ni^{2+} and Zn^{2+} toxicity in the Ni and Zn BLM, respectively. pH_k is the pH of solution *k*. $K_{CaBL,Ni}$ and $K_{CaBL,Zn}$ are the stability constants for binding of Ca^{2+} to the Ni and Zn biotic ligand, respectively. $K_{MgBL,Ni}$ and $K_{MgBL,Zn}$ are the stability constants for binding of Mg^{2+} to the Ni and Zn biotic ligand, respectively. $K_{HBL,Zn}$ and $K_{HBL,Pb}$ are the stability constants for binding of H^{+} to the Zn and Pb

biotic ligand, respectively. $K_{NaBL,Zn}$ is the stability constants for binding of Na^+ to the Zn biotic ligand. $\{Mg^{2+}\}_k$, $\{Ca^{2+}\}_k$, $\{H^+\}_k$, and $\{Na^+\}_k$ are the WHAM VII predicted chemical activities of Mg^{2+}, Ca^{2+}, H^+ and Na^+ in test solution *k* (mol/L), respectively. The MMBM is visualised in Figure 7.2.

Model parameters of the MMBM, i.e. intrinsic sensitivities ($Q50_{Ni2+,}$ $EC50^*_{Zn2+}$ and $EC50^*_{Pb2+}$) and the slopes (β_{Ni2+}, β_{Zn2+} and β_{Pb2+}) in Equation 7.7, were calibrated on all toxicity data from the individual metal treatments using non-linear least square fitting in R 3.1.10. However, biotic ligand stability constants for Mg^{2+}, Ca^{2+}, Na^+, and H^+ (K_{MgBLMe}, K_{CaBLMe}, K_{NaBLMe}, and K_{HBLMe}, respectively) and pH slopes ($S_{pH,Me}$) were taken from the orginal *C. dubia* bioavailbility models (De Schamphelaere et al. 2006; Chapter 2 & 4). The MMBM was used to predict relative reproduction in the mixture treatments. The accuracy of the MMBM for predicting chronic metal mixture toxicity was evaluated relative to the performance of the MMBM for predicting the individual metal toxicity. All MMBM model parameters are listed in Table 7.2.

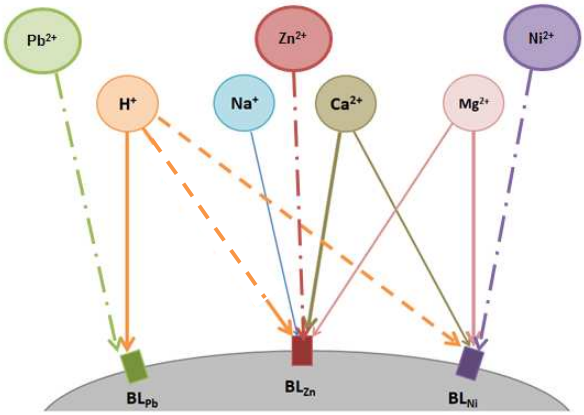

Figure 7.2. Visualisation of the chronic *Ceriodaphnia dubia* metal mixture bioavailability model (MMBM) for Ni, Zn and Pb (mixture) toxicity (Eq. 7.7). Cations (H^+, Na^+, Ca^{2+} or Mg^{2+}) compete (arrows) with Pb^{2+}, Zn^{2+}, or Ni^{2+} at the biotic ligand site (BL_{Pb}, BL_{Zn}, and BL_{Ni}, respectively). The thickness of the arrows is an indication of the relative importance of the competition of a cation relative to each of the metals (e.g. Mg^{2+} is the most important competitor at the Ni biotic ligand site). The competitive strength is defined by the value of the stability constant (e.g. log K_{CaBLNi}; see Table 7.2). The dashed line indicates that the effect of pH on Ni and Zn toxicity is not modelled as an unidentate biotic ligand site competition effect, but as a log-linear pH effect superimposed on the competition effects of Ca^{2+} and Mg^{2+}.

Table 7.2. Model parameters of the chronic *Ceriodaphnia dubia* metal mixture bioavailability model for Ni-Zn-Pb mixtures

		Ni	Zn	Pb
Fixed model parameters	Log K_{MgBLMe}	3.57[a]	2.69[b]	-
	Log K_{CaBLMe}	3.53	3.22	-
	Log K_{NaBLMe}	-	1.90	-
	Log K_{HBLMe}	-	-	7.6[c]
	$S_{pH,Me}$	0.8587	0.737	-
Calibrated model parameters[d]	$Q50_{Me2+}$	0.969±0.043	0.974±0.027	-
	$EC50^*_{Me2+}$ (nmol/L)	-	-	5.18±0.57
	β_{Me2+}	2.42±0.52	4.09±0.97	2.67±0.62

[a] Model parameters originating from the chronic Ni *C. dubia* bioavailability model: De Schamphelaere et al. (2006)
[b] Model parameters originating from the chronic Zn *C. dubia* bioavailability model: Chapter 4
[c] Model parameters originating from the chronic Pb *C. dubia* biotic ligand model: Chapter 2
[d] Calibrated MMBM model parameters ±standard deviation

Table 7.3 7d-Effective concentrations & slopes of individual dose response curves for Ni, Zn, and Pb observed in the different reproductive toxicity test with *Ceriodaphnia dubia*

Experiment	Test ID	$EC50_{Zndiss}$ (µg/L)	β_{Zndiss}	$EC50_{Nidiss}$ (µg/L)	β_{Nidiss}	$EC50_{Pbdiss}$ (µg/L)	β_{Pbdiss}	$EC50_{Zn2+}$ (nmol/L)	β_{Zn2+}	$EC50_{Ni2+}$ (nmol/L)	β_{Ni2+}	$EC50_{Pb2+}$ (nmol/L)	β_{Pb2+}
pH	Brisy pH base	225±14	5.33±1.72	39±3	10.6±5.5	135±10	4.75±2.09	1430±102	4.82±1.57	344±26	10.2±5.3	16±2	3.62±1.56
	Brisy pH 8	56±6	2.30±0.56	12±1	3.55±0.84	296±25	3.82±0.95	198±24	2.02±0.49	75±6	3.55±0.85	11±1	3.12±0.78
Ca	Brisy Ca base	130±11	3.62±1.07	24±2	2.89±0.54	114±8	5.74±1.59	730±69	3.23±0.96	206±14	2.80±0.52	11±1	4.63±1.29
	Brisy Ca 2mM	242±28[a]	11.3±3.9	30±2	4.70±0.92	171±8	7.58±3.26	1641±201[a]	10.4±3.6	268±18	4.60±0.90	30±2	6.03±2.46
Natural water	Brisy nat base	211±8	10.2±3.6	21±3	1.63±0.39	153±11	5.35±2.86	1345±56	9.11±3.23	181±29	1.59±0.38	19±2	3.57±1.59
	Ankeveen	145±6	10.7±2.8	23±2	2.84±0.60	312±10	15.1±22.4	470±24	8.99±2.35	126±10	2.65±0.54	8.7±0.3	11.5±16.0

[a] Because of the steepness of the dose response, no reliable EC50, EC10, and EC20 could be calculated for Zn for the 2 mM Ca test water with the log-logistic dose response. The EC50 was derived from the regression between the observed effect (%) at the 2 concentrations encompassing the 50% effect level and the log filtered concentration. Reported standard deviation is the difference between the 2 concentrations encompassing the 50% effect level, divided by two times 1.96. Reported slope is the fitted slope of the log-logistic dose response curve (Equation 7.3) wherein the $EC50_{Zndss}/EC50_{Zn2+}$ was fixed at 242 µg/L/1641 nmol/L, respectively.

7.3 Results & Discussion

7.3.1 Toxicity and bioavailability of individual Ni, Zn and Pb

The validity criteria for the control performance of the standard test protocol (USEPA 2002a) were met in all waters (Table D.1 in Appendix D). The average control reproduction in the different test waters ranged between 21 and 26 juveniles per parent animal. The dose response data of the individual metal exposures are shown in Figure 7.3. The corresponding EC50s are summarised in Table 7.3 and EC20s and EC10s are given in Appendix D (Table D.2). The individual toxicity of Ni, Zn, and Pb in the three Brisy base waters differed less than 2-fold between the different experiments: $EC50_{Ni2+}$ ($EC50_{Nidiss}$) ranged between 181-344 nmol/L (21-39 µg/L), $EC50_{Zn2+}$ ($EC50_{Zndiss}$) ranged between 730-1430 nmol/L (130-225 µg/L) and $EC50_{Pb2+}$ ($EC50_{Pbdiss}$) ranged between 11-19 nmol/L (114-153 µg/L). This is in line with the inter-test variability of chronic toxicity of these metals reported for *C. dubia* (De Schamphelaere et al. 2006; Chapter 2 & 4).

Individual Ni, Zn, and Pb toxicity, expressed as $EC50_{Mediss}$, varied at least 3-fold in the different test media. Dissolved Zn toxicity increased 3-fold when pH increased from pH 7 to 8. A similar effect of pH on dissolved Zn toxicity to *C. dubia* was observed in Chapter 4. Dissolved Ni toxicity increased 4-fold with increasing pH. This is in line with the trend of increasing Ni toxicity with increasing pH that was earlier reported for *C. dubia* (De Schamphelaere et al. 2006). High pH was protective against Pb toxicity as was previously observed (Chapter 2). Increasing Ca concentrations decreased dissolved Zn and Pb toxicity by 1.9- and 1.5-fold, respectively. The protective effects of Ca on Zn toxicity have been previously reported for daphnids (Heijerick et al. 2005) and it has been suggested that disruption of the Ca homeostasis is an important mechanism of chronic Zn toxicity for daphnids (Muyssen et al. 2006). A protective effect of Ca on chronic Pb toxicity to *C. dubia* was previously not observed by Mager et al. (2011). However, in the same natural Brisy water as in the present study Ca was shown to have minor protective effects on Pb toxicity (Chapter 2). Ca had only a minor effect on dissolved Ni toxicity (i.e. $EC50_{Zndiss}$ was 1.25-fold higher in the Ca 2mM medium). In comparison, dissolved Ni toxicity to *D. magna* decreased 1.7-fold with increasing Ca in the same Ca range as considered in the present study (Deleebeeck et al. 2008).

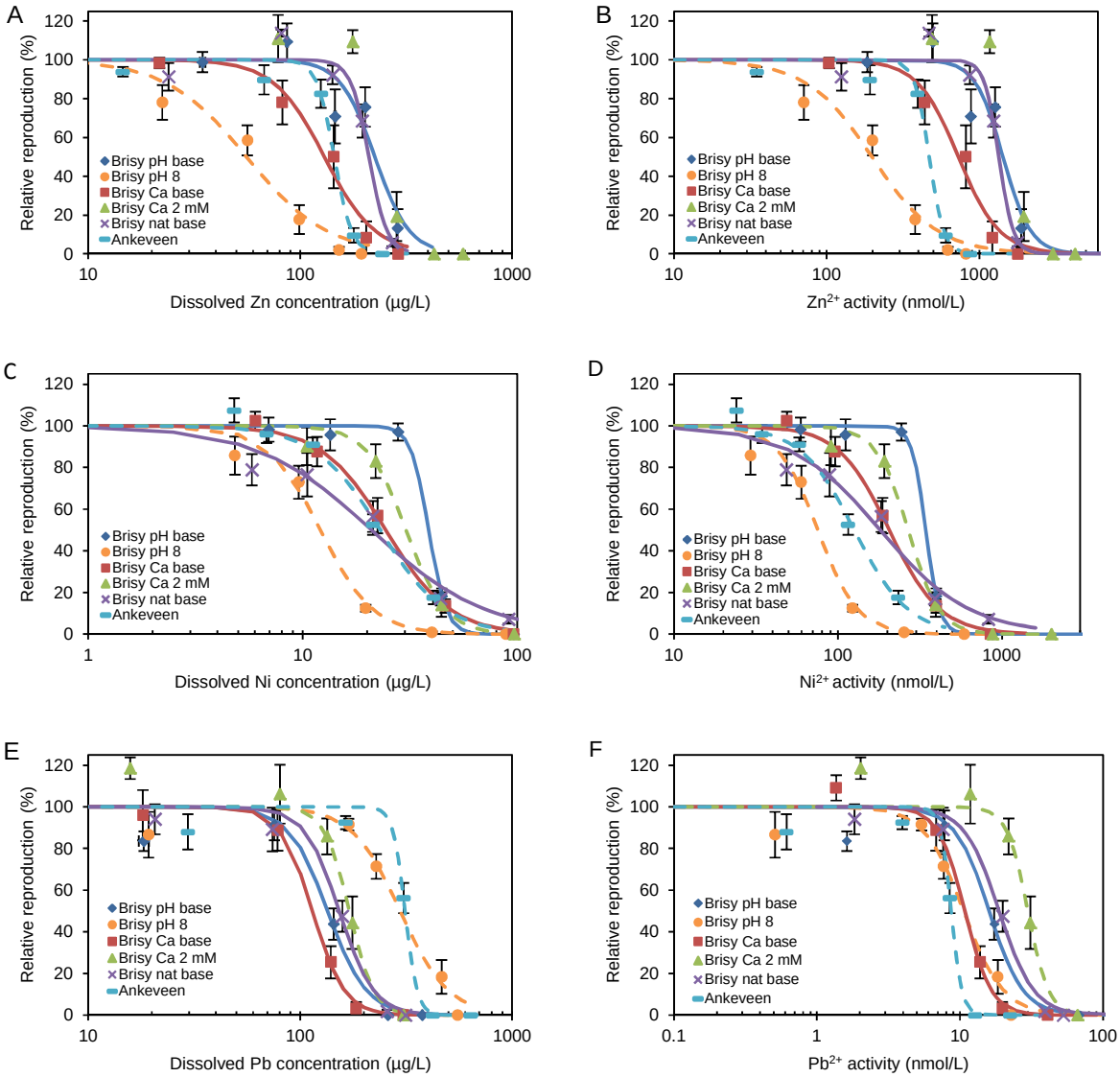

Figure 7.3. Dose response data of individual 7d-toxicity of Zn (A, B), Ni (C, D), and Pb (E, F) to *Ceriodaphnia dubia* in the individual metal exposures. Left panels show data based on dissolved concentrations, right panels based on calculated free ion activities.Data points are the mean reproductions relative to the control reproduction in the Brisy pH base (◊), Brisy pH 8 (○), Brisy Ca base (■), Brisy Ca 2 mM (▲), Brisy nat base (x) and Ankeveen (—) test waters. Plotted error bars denote standard errors. Lines are fitted log-logistic dose response curves (DRC; Equation 7.3; colours of the curves accord with those of the symbols). Full lines represent DRC of the Brisy base waters, dashed lines those of the other waters.

7.3.2 Objective 1: effect of metal concentration ratio on interactive effects

The measured dissolved metal concentration ratios in the *equitoxic* and *environmental mixture rays* are plotted together with those observed in the Dommel water basin in Figure 7.1. For both mixture rays, Zn:Ni concentration ratios fell within the 5^{th}-95^{th} percentile of the Zn:Ni ratios observed in the Dommel monitoring dataset. Also, the Zn:Pb ratios in the *environmental* mixture treatments fell mostly within the 5^{th}-95^{th} percentile of those observed in the Dommel monitoring dataset. However, for some mixture treatments in the *environmental* rays Zn:Pb ratios were lower than targeted, probably due to geochemical effects occurring in the test solutions. In contrast, the Zn:Pb concentration ratios in the *equitoxic* mixture treatments were always lower than the 5^{th} percentile of the Zn:Pb ratios observed in the Dommel monitoring dataset. Based on this comparison, Zn:Pb metal concentration ratios in the equitoxic mixture treatments are not representative for the observed Zn:Pb ratio in the Dommel river basin. However, metal concentrations may vary between water basins. Hence, this picture can potentially be different if the metal ratios are compared to concentrations measured in another river basin.

Since the free metal ion is generally considered as the most bioavailable and thus most toxic metal fraction, the description of mixture effects below will mainly focus on the analysis based on free metal ion activities. However, results of the analysis based on dissolved metal concentrations were in general similar. The observed RR and CA-predicted and IA-predicted RR in the Ni-Zn-Pb mixture treatments are shown as a function of the sum of TU_{Me2+} in Figure 7.4 and as a function of sum of TU_{Mediss} in Figure D.1. The observed RR in the equitoxic mixture ray (filled squares) were mostly situated above the dose response curves of the individual metals in all waters, indicating a general trend towards antagonistic interactions relative to the CA model. Predicted RR of the IA model (filled triangles) were in general closer to the observed RR than those of the CA model. The statistical analysis showed that the mixture interactions in the equitoxic ray in all waters were significant antagonistic relative to the CA model (Table 7.4). The interactions in the equitoxic mixture ray were mostly non-interactive relative to the IA model. In the environmental mixture rays, the mixtures acted non-interactively in all waters relative to both the IA model and the CA model.

Table 7.4. Observed type of interactive effect of Ni-Zn-Pb mixtures on 7d-reproductive toxicity to *Ceriodaphnia dubia* in the equitoxic ray and environmental ray following the statistical analysis of the mixture interactions[a].

		Equitoxic mixture ray				Environmental mixture ray			
		Concentration addition		Independent action		Concentration addition		Independent action	
	Test water ID	Me_{diss}[c]	Me^{2+}[d]	Me_{diss}[c]	Me^{2+}[d]	Me_{diss}[c]	Me^{2+}[d]	Me_{diss}[c]	Me^{2+}[d]
pH experiment	Brisy pH base	A	A	NI	NI	A	NI	NI	NI
	Brisy pH 8	A	A	A	A	NI	NI	NI	NI
Ca experiment	Brisy Ca base	A	A	NI	NI	A	A	NI	NI
	Brisy Ca 2 mM	A	A	NI	NI	NI	NI	NI	NI
Natural water experiment	Brisy nat base	A	A	NI	NI	NI	NI	NI	NI
	Brisy Ankeveen	A	A	NI	NI	NI	NI	NI	NI

[a] All parameters of the fitted models are listed in Table D.3-8.; Figure D.2-3
[b] Expressed as relative Toxic $Unit_{Mediss}$ (TU_{Mediss})
[c] Analysis of mixture interactions based on dissolved concentrations.
[d] Analysis of mixture interactions based on free ion activities
[e] Expressed as measure dissolved metal concentration ratios
A= antagonistic; NI= non-interactive

For most waters, different interactive effects were observed between the *equitoxic* ray and the *environmental* ray when evaluated relative to the CA model. Thus, the metal concentration ratio at which Ni, Zn and Pb are present in the mixture affects the type of interactive effect observed. A concentration ratio dependency of interactive metal mixtures effects has been reported for several terrestrial species (Jonker et al. 2004; 2005; Qui et al. 2001). The occurrence of concentration ratio dependent metal mixture effects may implicate that the evaluation of interactive effects based on equitoxic metal concentration ratios, which are often used in metal mixture studies, are not always representative for realistic mixture scenarios. However, when mixture effects where evaluated relative to the IA model, the observed interactive effects in the *equitoxic* vs *environmental* ray differed only for one water.

7.3.2 Objective 2: effect of water chemistry on interactive effects

Water chemistry has been shown to influence the type of interactive effects observed in a mixture (e.g. Versieren et al. 2014, Naddy et al. 2015). Our results suggest that water chemistry may influence mixture effects to some extent. However, shifts in interactive effects due to varying water chemistry occurred only in one water and this only in the *equitoxic* mixture ray and only relative to the IA model. When pH increased from pH 7 to pH 8, the interactive effects in the *equitoxic* rays of the pH experiment shifted from non-interaction to antagonism when evaluated relative to the IA model. Up to a 4-fold difference between the IA-predicted RR and

observed RR was observed in the Brisy pH 8 water (Figure 7.4.B). This shift in interactive effect with increasing pH can potentially be explained using the biotic ligand theory. At low pH, H^+ outcompetes Me_2 and Me_3 cations for binding at the biotic ligand site of $Me_{1.}$ However, an increase in pH decreases the competition of H^+ for binding at the biotic ligand site of Me_1 and the competitive effects between the metals become more important, possibly resulting in the observed antagonistic effects at high pH. In this reasoning it is assumed that binding of Me_2 or Me_3 at the biotic ligand site of Me_1 does not result in toxicity, although it is not known if this assumption holds true. Additionally, a similar shift in interactive effects was not observed in the natural water experiment, although the pH in the Ankeveen water was also around 8. As a consequence, the mechanisms underlying this shift in interactive effects remain unclear.

It has been reported that mixture interactions are not always reproducible when experiments are replicated (Cedergreen et al. 2007). However, the observed interactive effects in all Brisy base waters in the *equitoxic* ray (antagonistically relative to the CA model; non-interactively relative to the IA model) were in line with the interactions previously observed for the ternary Ni-Zn-Pb mixture in this water (Chapter 6). Additionally, the observed mixture effects of the *environmental* mixture rays in all Brisy base mediums were the same for the IA model. In the *environmental* rays, in contrast, the mixture effect evaluated relative to the CA model in the Brisy base water of the Ca experiment differed from those observed in the other experiments. However, the antagonism observed in the Brisy base water in the Ca experiment was only slightly significant ($p=0.03$) and overall the difference in CA-predicted RR and observed RR did not differ that much between the Brisy base waters in the different experiments (open symbols in Figure 7.4.A, C & E). This suggest that overall the joint effects of Ni-Zn-Pb mixtures are reproducible.

Since the IA and CA model are not nested, a statistical comparison between both models cannot be made. However, the goodness of fit of both models can be quantitatively evaluated using for instance the Aikake Information Criterion (AIC). Generally, the IA model fitted the data better than the CA model, i.e. the AIC of the IA model was almost always lower than these of the CA model (Table D.3-8). This in line with the observations earlier made in Chapter 6 (albeit in a single medium) and it has been suggested that this could be explained by the different modes of action of Ni, Zn and Pb for daphnids.

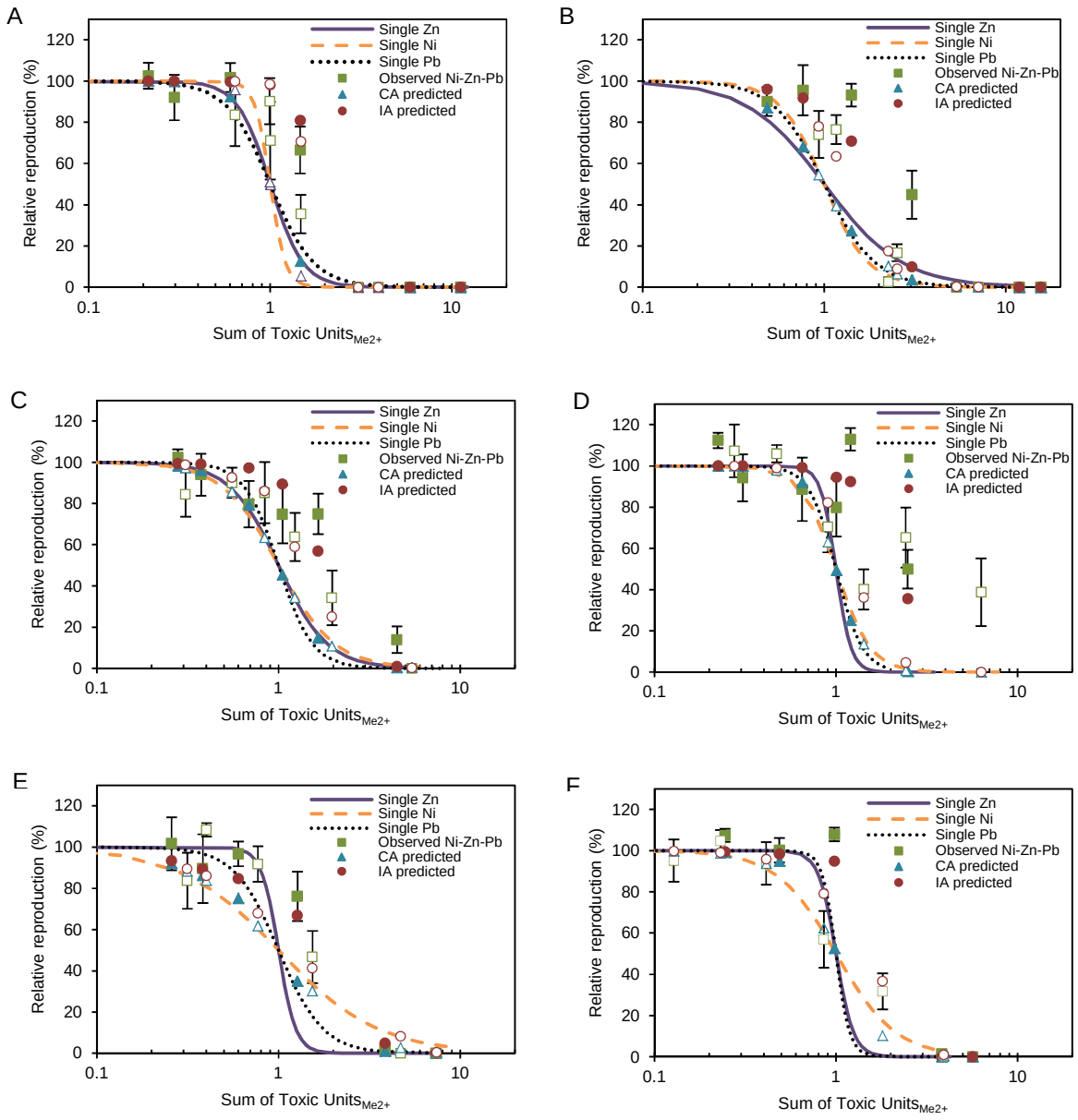

Figure 7.4. Observed and predicted 7d-relative reproduction (%) of *Ceriodaphnia dubia* in the mixture combinations of the Ni-Zn-Pb mixture as a function of sum toxic units based on free ion activities in the Brisy pH base (A), Brisy pH 8 (B), Brisy Ca base (C), Brisy Ca 2 mM (D), Brisy nat base (E) and Ankeveen (F) waters. Symbols are denoted as follows: observed reproduction (squares), concentration addition predicted reproduction (circles; Equation 7.4), independent action predicted reproduction (triangles; Equation 7.5). Filled symbols denote observed and predicted reproduction in the equitoxic mixture ray. Open symbols denote observed and predicted reproduction in the environmental mixture ray. Predictions are based on the parameters ($EC50_{Me2+}$ and β_{Me2+}) of the individual concentration-response curves of Ni, Zn, and Pb (Table 7.3). Lines represent the individual log-logistic concentration response curve (Equation 7.3) of Ni (dashed line), Zn (full line), and Pb (dotted line) in the corresponding test waters.

7.3.2 Objective 3: Validation of the metal mixture bioavailability model

7.3.2.1 Prediction performance of the individual chronic C. dubia BLMs

To be able to integrate the individual chronic *C. dubia* BLMs for Ni, Zn, and Pb (De Schamphelaere et al. 2006; Chapter 2 & 4) in a MMBM, it is crucial to demonstrate that these BLMs can reproduce the $EC50_{Mediss}$ of the individual metal treatments in the test waters considered in this study. This is because large over- or underestimations of $EC50_{Mediss}$ would result in a considerable under- or overestimation of metal toxicity by the MMBM, respectively. The prediction performance of the individual BLMs is shown in Figure 7.5. All $EC50_{Nidiss}$, $EC50_{Zndiss}$ and $EC50_{Pbdiss}$ in the Ni, Zn, and Pb only exposures, respectively, were predicted within 2-fold error (Figure 7.5). This is in line with the prediction capacities reported for the bioavailability models of these metals (De Schamphelaere et al. 2006; Chapter 2 and 4) and supports the incorporation of the individual chronic *C. dubia* BLMs in a chronic MMBM.

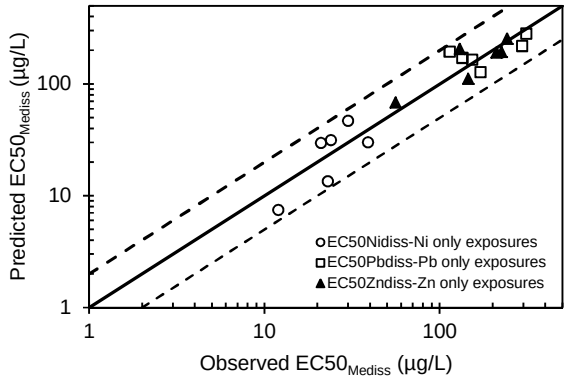

Figure 7.5. Predicted versus observed median effective concentration (7d-$EC50_{Mediss}$, expressed as µg dissolved Me/L) for *Ceriodaphnia dubia* in the indivdiual Ni (circles; predicted with the *C. dubia* Ni BLM; De Schamphelaere et al. (2006); Eq. 3.3), Pb (squares; predicted with the *C. dubia* Pb BLM; Chapter 2; Eq. 2.4), and Zn (triangles; predicted with the *C. dubia* Zn BLM; Chapter 4; Eq. 4.5) exposures. All BLM parameters are reported in Table 7.2. The dashed lines representa difference of a factor of 2 between the observed and predicted data. The full line represents a perfect fit between observed and predicted data.

7.3.2.2 Development & validation of the metal mixture bioavailability model

In the present study, we evaluate whether a relatively simple metal mixture bioavailability model that combines the existing chonic daphnid bioavailability models for the individual metals (De

Schamphelaere et al. 2006; Chapter 2 & 4) with the independent action model can accurately predict chronic toxicity of Ni-Zn-Pb mixtures to *C. dubia*. The IA model was selected as the basis to develop the MMBM, because it was observed that Ni-Zn-Pb mixture toxicity obeys the IA model rather than the CA model (Chapter 6 and present study). Furthermore, the individual *C. dubia* bioavailability models of Ni, Zn and Pb are relatively different in structure and the competing ions that they consider (discussed in Chapter 6). This all suggests that Ni, Zn and Pb have different modes of actions and bioavailability relations and supports the choice for an IA-based MMBM.

For modelling the metal interactions at the biotic ligand sites, we assumed that metals bind at multiple biotic ligand sites (Figure 7.2). Metal (mixture) toxicity was related to the concentration of free metal ion binding to its metal-specific BL. The chronic MMBM is in that respect comparable to the metal mixture model previously used by Santore & Ryan (2015) and Versieren et al. (2014). However, in contrast to the models of these authors, competition between Ni^{2+}, Zn^{2+} and Pb^{2+} for binding at the biotic ligand sites was in the present study not allowed, and it is assumed that each metal binds at its own biotic ligand (BL) site. This assumption was based on the observation that Ni, Zn, and Pb do not interact with each other in most binary and ternary mixture combinations of Ni, Zn, and Pb relative to the IA reference model (Chapter 6).

Traditionally, the individual BLMs have been used to predict ECx (Di Toro et al. 2001; De Schamphelaere et al. 2002; see Figure 7.5). In contrast, the MMBM developed here predicts toxicity expressed as the relative reproduction of *C. dubia*, i.e. reproduction relative to a control situation. The structure of the MMBM allows the prediction of the reproductive toxicity of Ni, Zn, and Pb when they are combined in a mixture, as well as the toxicity of these metals when they are individually present. The MMBM parameters were calibrated on toxicity data of the individual metal treatments (Table 7.2). Then, the MMBM was used to predict relative reproduction in the mixture treatments. Hence, the performance of the chronic MMBM for predicting metal mixture toxicity was evaluated relative to the toxicity predictions made for the individual metals. The latter is in our opinion a more correct comparison than when prediction capacities of the MMBM for mixture toxicity would be compared to a free metal or dissolved metal based independent action model not taking into account bioavailability effects. This is because it has been established that free metal ion concentrations alone are not a good predictor of metal toxicity

(e.g. reviewed by Campbell 1995; Paquin et al. 2002; see also Chapter 2-4). Hence, from this it is logical that a metal mixture model that does not take into account potential competition effects at the biotic ligand sites will result in less accurate predictions of metal mixture toxicity under varying water chemistries.

The MMBM predicted the RR of 85% of the mixture treatments with less than 20% error, while the RR in 73, 83, and 70% of the individual Zn, Ni, and Pb treatments was predicted (Figure 7.6; Table 7.5). Root mean square errors were 16, 18, 17, and 23 for the mixture, individual Zn, individual Ni, and individual Pb treatments, respectively. Thus, the MMBM predicted chronic toxicity of the ternary Ni-Zn-Pb mixture at least equally accurately as the toxicity observed in the individual metal treatments. The latter indicates that the MMBM can be used to predict Ni-Zn-Pb mixture toxicity under varying water chemistries.

Table 7.5. Prediction errors (%) of the metal mixture bioavailability model (MMBM, Eq. 7.7), expressed as the difference between observed and predicted 7d-relative reproduction of *Ceriodaphnia dubia*, in the Zn only, Ni only, Pb only and Ni-Zn-Pb mixture exposures and root mean square error (RMSE)

	Zn only	Ni only	Pb only	Mixtures
Mean prediction error (%)	-1.68	-1.15	2.31	3.00
Median prediction error (%)	-1.88	-2.03	-3.75	-0.01
% predicted within 5% error	33	23	20	38
% predicted within 10% error	47	47	40	63
% predicted within 20% error	73	83	70	85
RMSE	18	17	23	16

However, a tendency to overestimate mixture toxicity effects was observed in some treatments. For instance, relative reproduction in the Brisy pH 8 water, the only water in which Ni-Zn-Pb mixtures interacted significantly antagonistically relative to the IA model (Table 7.4), was almost consistently underestimated by the MMBM. This may suggest that the assumption on the absence of competition between the metals for binding at their respective BL-sites is too simple. Additional mixture toxicity testing in test waters with low cationic competition, e.g. high pH or low Ca, might identify competitive effects between metals occurring at the biotic ligand sites and improve the metal mixture bioavailability modelling. A better understanding of competitive interactions occurring at the bioaccumulation level might increase our understanding of

interactive effects observed at the life-history level and further improve metal mixture bioavailability modelling.

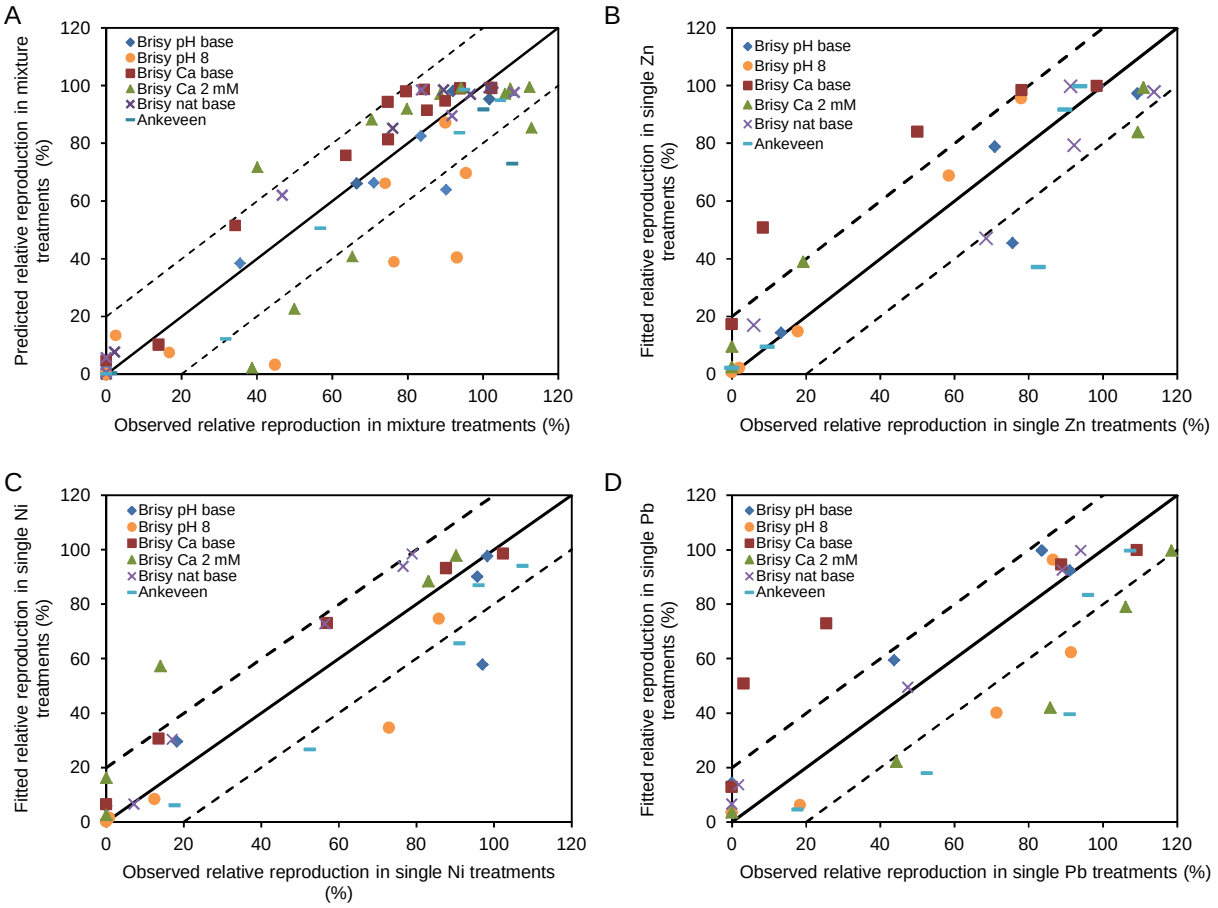

Figure 7.6. MMBM predicted versus observed 7d-relative reproduction of *Ceriodaphnia dubia* in the Ni-Zn-Pb metal mixture treatments (A), individual Zn treatments (B), individual Ni treatments (C) and individual Pb treatments (D) in the different test waters . The full line represents a perfect fit between observed and predicted data. The dashed line represents a difference of 20% between the observed and predicted data. The symbols are designated as follows: ◊ Brisy pH base, • Brisy pH 8, ■ Brisy Ca base, ▲ Brisy Ca 2 mM, × Brisy nat base, — Ankeveen

The MMBM developed in the present study implicitly assumes that the slopes of concentration response curves are independent of water chemistry. However, the validity of this assumption is at the moment unclear. Despite decades of toxicological research, slopes of concentration response curves and the factors that might influence them have untill now received little

attention. The observed slopes in the waters considered in this chapter are relatively variable (Table 7.3) between the waters of the different experiments, but also between the Brisy base waters (i.e. the waters with the same physico-chemistry) in the different experiments. This suggests that also between test variability might also be an important factor in the slope of the concentration response curve. Hence, untill a better understanding of the mechanisms influencing the slopes of the concentration response curves, the assumption of the MMBM that the water chemistry does not influence the slope of the concentration response curves seems to be fair.

Recently, several metal bioavailability approaches have been shown to successfully predict acute mixture toxicity to a variety of aquatic organisms (Farley et al. 2015). However, in risk assessment frameworks mainly chronic exposures are of importance. Therefore, chronic mixture datasets describing full water chemistries are needed to evaluate chronic metal mixture bioavailability models (Van Genderen et al. 2015; Meyer et al. 2015a). The present study is one of the first to evaluate a bioavailability based modelling approach to predict mixture toxicity during chronic exposure (see also Santore and Ryan 2015; Farley et al. 2015). Overall, our results suggest that relatively simple chronic MMBMs can be used to account for the effects of water chemistry on metal mixture toxicity during chronic exposure and that these type of models could potentially be used in metal risk assessment frameworks. The application of MMBMs in metal risk assessment frameworks will be addressed in Chapter 8.

8

Chronic metal mixture toxicity to *Ceriodaphnia dubia*: implications for ecological risk assessment

8. Chronic metal mixture toxicity to *Ceriodaphnia dubia*: implications for ecological risk assessment

8.1 Introduction

Although metal mixture toxicity has been investigated for several decades, until now no clear patterns have emerged from these metal mixture studies due to the variability in the observed interactive effects (reviewed by Norwood et al. 2003; Vijver et al. 2011). Furthermore, until recently most studies investigated interactive effects during short-term exposure (but see e.g. Naddy et al. 2015; Norwood et al. 2013), while in European risk assessment frameworks the preference is given to toxicity data originating from chronic exposures (EC 2003). All of this currently hinders the integration of metal mixture toxicity into risk assessment procedures (Van Genderen et al. 2015; Meyer et al. 2015). At the moment, most risk assessment frameworks still evaluate environmental risks of aquatic exposure on a substance-by-substance basis (Backhaus & Faust 2012). However, it has been anticipated that in future risk assessment procedures it will be necessary to account for mixture toxicity effects (SCHER 2009; CEU 2009).

The present doctoral thesis focused on investigating the interactive effects of Ni, Zn, and Pb mixtures on chronic reproductive toxicity to daphnids. Overall, the results suggested that chronic metal mixture effects can be as variable as acute metal mixture effects. The interactive effects were shown to depend on the considered mixture reference model (concentration addition (CA) vs. independent action (IA); Chapter 5-7), the test species (*Daphnia magna* vs. *Ceriodaphnia dubia*; Chapter 6), the applied metal concentration ratio (environmental vs equitoxic ray; Chapter 7), the concentrations of the individual metals (i.e. effect size dependency; Chapter 5) and the metal combination (Pb-Zn vs. Ni-Zn mixtures in Chapter 6). Furthermore, metals compete for DOC binding sites and as such also the expression of exposure (dissolved concentrations vs. free ion activities) was shown to determine the observed interactive effects (Chapter 6). Additionally, the water chemistry of the receiving water can influence interactive effects (e.g. pH in Chapter 7). Thus, at first sight, even for a restricted taxonomical scope and number of metals no clear patterns in chronic metal mixture toxicity have emerged from the present study either.

In the present chapter, we therefore combined all metal mixture toxicity data for *C. dubia* from the present study (Chapter 6 and 7) and one additional study (Nys et al. 2015) in a meta-

analysis. Here, we evaluated the implications of these data for metal mixture risk assessment frameworks. Moreover, the following three risk assessment related questions were addressed : I) are mixture effects important?; II) is the CA model a conservative model for chronic metal mixture toxicity?, III) which of the two commonly applied mixture reference models (CA or IA) describes metal mixture toxicity most accurately?

The necessity of a metal mixture risk evaluation scheme evolves around the question whether chronic metal mixture toxicity is important: i.e. are observed metal mixture effects larger than the effects of the individual metals in the mixture? This question might give us a first idea whether there is an actual need to account for potential metal mixture effects or if the existing risk assessment frameworks for the individual metals are sufficient to protect aquatic communities against multi-metal exposures.

Because the CA model is generally considered to be the most conservative reference model (Cedergreen et al. 2008; Kortenkamp et al. 2009), it has been proposed as a first tier in mixture risk assessment processes (Backhaus & Faust 2012). Therefore, we investigated whether this assumption also holds true for metal mixture toxicity to *C. dubia*, especially at the low effect concentrations that are of regulatory relevance (i.e. at concentrations below or around 10% effect). However, the most conservative model is not necessarily the model that most accurately predicts metal mixture toxicity. Identifying the best model would allow a decision on which method is most appropriate to use in a higher tier of a tiered risk evaluation framework.

Finally, we proposed a possible tiered approach for evaluating metal mixture risk. The proposed tiered metal mixture risk assessment scheme was used to evaluate risks of Ni, Zn, and Pb exposures in the Dommel water basin (The Netherlands)

8.2 Materials and methods

8.2.1 Data selection

The metal mixture toxicity data of Chapter 6 and 7 of the present doctoral thesis were included in the meta-analysis (Table 8.1). Additionally, we also included data of a study conducted in another project (Nys et al. 2015). In that project, we have investigated the interactive effects of the ternary Ni-Zn-Cu and quaternary Ni-Zn-Cu-Cd mixtures on *C. dubia* reproductive toxicity. Two

experiments were conducted. In the first experiment, toxicity of the quaternary Ni-Zn-Cu-Cd mixture was investigated, while in the second experiment the toxicity of both the ternary Ni-Zn-Cu and quaternary Ni-Zn-Cu-Cd mixtures was investigated.

Table 8.1. Overview of mixture combination included in the meta-analysis of the effects of metal mixture toxicity on reproduction of *Ceriodaphnia dubia*.

Mixture combination	Number of experiments	Number of treatments	Reference
Ni-Zn	1	36	Chapter 6
Pb-Zn	1	36	Chapter 6
Ni-Pb	1	36	Chapter 6
Ni-Zn-Pb	7	77	Chapter 6, Chapter 7
Ni-Zn-Cu	1	15	Nys et al. 2015
Ni-Zn-Cu-Cd	2	10	Nys et al. 2015
Total	13	210	-

8.2.2 General data-treatment used in all mixture studies

The mixture data from the different chapters of this doctoral thesis were all analysed in a similar way using the same methods and assumptions for speciation calculations in WHAM VII (Tipping et al. 2011; assumptions Chapter 5-7), and concentration response fitting of the individual metal exposures (2 parameter log-logistic concentration response model). The data reported in Nys et al. (2015) were originally evaluated based on slightly different speciation assumptions. However, free ion activities were recalculated using the assumptions described in Chapter 5-7 and the metal responses, expressed as free ion activities, were refitted and reported in Nys et al. (submitted). While in previous chapters metal mixture toxicity was usually expressed as relative reproduction (i.e. the relative response of the mixture; RR_{mix}), in the present chapter mixture toxicity will be expressed as relative mixture effects, because this expression of toxicity is preferred in regulatory contexts. The relative mixture effect for every metal treatment, i.e. the effect of the mixture relative to the control (RE_{mix}, %), was calculated using Equation 8.1.

$$RE_{mix} = 100 - RR_{mix} \quad (8.1)$$

Parameters of the individual metal concentration response curves (β_{Mediss} and $EC50_{Mediss}$ or β_{Me2+} and $EC50_{Me2+}$) of each mixture experiment were taken from the respective Chapters or from Nys et al. (submitted).

8.2.3 Importance of metal mixture effects

The effects of the individual metals was calculated by combining the 2-parameter log-logistic concentration response model with Equation 8.1 (Equation 8.2):

$$RE_{ind\ Me_i,pred} = 100 - \left(\frac{100}{1+\left(\frac{x_{Me_i}}{EC50_{Me_i}}\right)^{\beta_{Me_i}}} \right) \quad (8.2)$$

In Equation 8.2, $RE_{ind\ Mei,pred}$ is the individual predicted effect (%) of metal *i*. x_{Mei} is the dissolved concentration or free ion activity of metal *i*. $EC50_{Mei}$ is the 50% effective concentration of metal *i*, either expressed as dissolved concentration ($EC50_{Mediss}$) or as free ion activity ($EC50_{Me2+}$). β_{Mei} is the slope parameter of the dose response of metal *i*, based on dissolved concentrations (β_{Mediss}) or free ion activities (β_{Me2+})

To evaluate the importance of metal mixture effects, we tested whether the deviation ($Dev_{mix-ind}$; Eq. 8.3) between the observed mixture effects ($RE_{mix,obs}$) and the predicted effect of the most toxic metal in the mixture (max $RE_{ind,pred}$) was significantly larger than 0 using a one-tailed t-test (α=0.05) in R Version 3.1.2.

$$Dev_{mix-ind} = RE_{mix,obs} - \max RE_{ind\ Mei,pred} \quad (8.3)$$

8.2.4 Prediction performance of the mixture reference models

For every dataset, the parameters of the individual concentration response curves ($EC50_{Me_i,diss}$ and $\beta_{Me_i,diss}$/ $EC50_{Me_i,2+}$ and $\beta_{Me_i,2+}$) reported in the respective chapter or study (Table 8.1) were used to predict the response in the metal mixture treatments following both the CA (Eq. 8.4) and IA (Eq. 8.5) mixture reference model.

$$\sum_{i=1}^{n} \frac{x_{Me_i}}{EC50_{Me_i} \times \left(\frac{100-RR_{mix,pred}}{RR_{mix,pred}}\right)^{\frac{1}{\beta_{Me_i}}}} = 1 \quad (8.4)$$

$$RR_{mix,pred} = 100 \times \prod_{i=1}^{n} \left(\frac{1}{1+\left(\frac{x_{Me_i}}{EC50_{Me_i}}\right)^{\beta_{Me_i}}} \right) \quad (8.5)$$

In Equation 8.4 and 8.5, *n* is the number of metals in the mixture, and $RR_{mix,pred}$ is the predicted mixture response relative to the control response (%). The non-linear equation of the CA model (Eq. 8.4) was solved for $RR_{mix,pred}$ using the generalized reduced gradient iterative solver function in Excel 2010. The performance of the two mixture reference models was evaluated by comparing the predicted RE_{mix} to the observed RE_{mix}, using the root mean squared error (RMSE). We evaluated if the overall prediction bias for both reference models was significant, by testing whether the overall deviation between the IA or CA predicted mixture effect and the observed mixture effect were significantly different from 0 using a one-sample t-test in R.

Equation 8.4 and 8.5 assume the absence of interactive effects between the metals (Jonker et al. 2005). Treatment specific deviations from non-interactivity were evaluated relative to both mixture reference models by comparing the CA and IA predicted relative mixture effect ($RE_{mix,pred}$) for every treatment, calculated with Equation 8.4 and 8.5, with the observed mixture effect ($RE_{mix,obs}$). In practice, $RE_{mix,pred}$ that were higher than the 95% confidence interval (CI) around the $RE_{mix,obs}$ were considered as a significant antagonism, while $RE_{mix,pred}$ that were lower than the 95% CI around the $RE_{mix,obs}$ were considered as a significant synergism (Sharma et al. 1999, Chapter 4).

8.2.5 Protectiveness of the CA model at low effect sizes

To evaluate the degree of protectiveness of the CA model at low effect sizes, mixture concentrations were first expressed as Toxic Units (TU) relative to the EC10 using Equation 8.6.

$$\sum TU_{EC10} = \sum_{i=1}^{n} \frac{x_{Me_i}}{EC10_{Me_i}} \qquad (8.6)$$

In Equation 8.6, $\sum TU_{EC10}$ is the sum of toxic units expressed relative to the 10% effective concentrations. $EC10_{Me_i}$ is the 10% effective concentration for metal *i* reported in the (Appendixes of the) respective Chapters or in Nys et al. (2015). The $EC10_{Me_i}$ was either expressed as dissolved metal concentration ($EC10_{Mediss}$) or free ion activity ($EC10_{Me2+}$). The $\sum TU_{EC10}$ was plotted as a function of the observed mixture effect. If the CA model is conservative at low effect sizes (i.e. at or below the 10% effect size) than the observed mixture effects for mixture treatments where $\sum TU_{EC10}<1$ should be maximum 10%.

To evaluate the level of conservativeness of the CA model at these low effect sizes, we calculated an $EC10_{TUEC10}$ for every mixture experiment by fitting the 2 parameter log-logistic concentration response model expressed as Equation 8.7 to the $\sum TU_{EC10}$ as expression of concentration and the observed RE_{mix}(%) as endpoint in Statistica 7 (Statsoft).

$$RE_{ind\ Me_i,pred} = 100 - \left(\frac{100}{1+\left(\frac{\sum TU_{EC10}}{EC50_{TU_{EC10}}}\right)^{\frac{\ln\left(\frac{1}{9}\right)}{ln\left(\frac{EC10_{TU_{EC10}}}{EC50_{TU_{EC10}}}\right)}}} \right) \quad (8.7)$$

If the 2 parameter log-logistic response model could not be fitted, the $EC10_{TUEC10}$ were derived from a linear relation between the observed effect (%) at the 2 concentrations encompassing the 10% effect level and the log filtered concentration. When different mixture rays were investigated in one experiment, a different $EC10_{TUEC10}$ was calculated for every ray. After this, six parametric distributions (normal, log-normal, exponential, logistic, weibull, and gamma) were fitted to all calculated $EC10_{TUEC10}$ for the different mixtures. The best fitting distribution was selected based on the Kolmogorov-Smirnov goodness-of-fit test statistic (Aldenberg et al. 2002). The median $EC10_{TUEC10}$ was calculated as the 50% percentile of the best fitting distribution. The sampling uncertainty was taken into account by parametric bootstrapping using the fitdistrplus package in R (Delignette-Muller & Dutang 2015).

The same analysis was done for the chronic mixture toxicity data of two other species. For *D. magna*, the data of chronic toxicity of Ni-Zn mixtures reported in Chapter 4 were used. For *P. subcapitata*, toxicity of the ternary Ni-Zn-Cu and quaternary Ni-Zn-Cu-Cd mixture was reported in Nys et al. (2015). The mixture data of these two species was treated as described above. The geometric mean $EC10_{TUEC10}$ for these species was combined with the geometric mean of $EC10_{TUEC10}$ for *C. dubia* in Figure 8.7.

8.3 Results & Discussion

The present meta-analysis combined in total 210 mixture treatments investigating the chronic mixture toxicity of 6 metal combinations of Ni, Zn, Pb, Cu, and Cd to *C. dubia.* A literature search revealed two additional studies investigating chronic mixture toxicity of Ni, Zn, Pb, Cu,

and/or Cd to *C. dubia*. Cooper et al. (2009) investigated the interactive effects in binary and ternary metal combinations of Cu, Zn, and Pb. Naddy et al. (2015) investigated the toxicity of ternary Cd-Cu-Zn mixtures. However, the mixture data reported in these studies were not considered in the present meta-analysis based on the following considerations: I) mixture toxicity in these studies was investigated in reconstituted waters without the addition of dissolved organic matter, which makes these mixture studies less representative for exposure situations in the natural environment; II) metal concentrations were measured as total concentrations, while dissolved concentrations are of relevance in metal risk assessment frameworks (e.g. ECI 2008; Van Sprang et al. 2009); and III) for the study performed by Cooper et al. (2009) it was unclear whether the mixture toxicity tests were performed concurrently with the individual metal treatments, while it is known that non-simultaneously testing can lead to erroneous conclusions about metal mixture toxicity (De Laender et al. 2009).

8.3.1 Importance of metal mixture toxicity

The observed mixture effect was in most mixture treatments larger than the predicted individual effect of the most toxic metal (62% of the mixture points are situated above the 1:1 line in Figure 8.1). Overall, the observed effect in the mixture was significantly higher than the predicted effect of the most toxic metal (mean $Dev_{mix\text{-}ind}$=1.92±1.06%, one tailed t-test: t=1.81, p=0.04). This indicates that chronic metal mixture toxicity is indeed important for *C. dubia* and that protecting ecosystems on a metal-by-metal basis, without accounting for possible mixture effects, could lead to an underestimation of the effects of exposures to multi-metal combinations in certain situations. However, in 38% of the mixture treatments the observed mixture effect was lower than the predicted individual effect of the most toxic metal, suggesting that antagonistic interactions may also occur in metal mixtures.

When the mixture treatments relevant for risk assessment were considered, i.e. the mixture treatments where the predicted individual effect of the most toxic metal was lower than 10% (data points at the left of the dashed line in Figure 8.1), the observed mixture effect was higher than the predicted individual effect of the most toxic metal for 60% of the treatments. Additionally, for 33% of these mixture treatments, the observed mixture effect was higher than 10%, although the effects of the individual metals in the mixture were less than 10% (Figure

8.1, red shaded area). Up to 35% mixture effect was observed in these treatments. The latter indicates that significant mixture effects can occur when combining metals below their EC10 levels. However, it has to be kept in mind that in other mixture treatments reproduction was higher than in the corresponding control treatment (i.e. negative mixture effects in Figure 1). This suggests that mixture effects of metals combined below their EC10 can be variable.

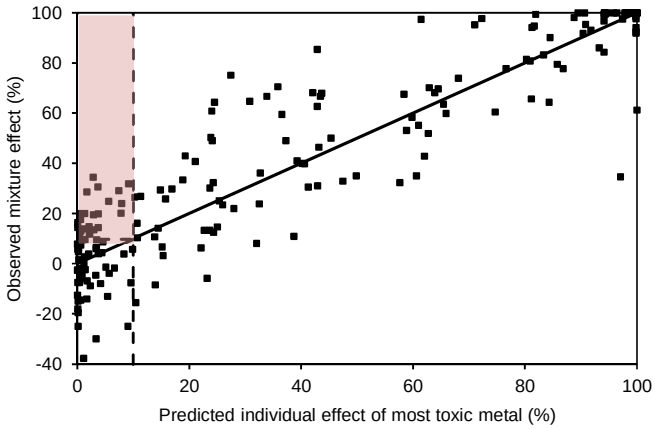

Figure 8.1. Observed mixture effect (%) plotted against the predicted individual effect of the most toxic metal in the mixture (%)for the reproductive toxicity of Ni, Zn, Pb, Cu, and Cd mixtures to *Ceriodaphnia dubia*. The diagonal line represents the situation where the observed mixture effect is equal to the predicted individual effect of the most toxic metal. The vertical dashed line denotes the situation where the most toxic metal is present at a concentration causing on itself less than 10%. The red box indicates the situations of relevance for risk assessments, i.e. situations where the predicted individual effect of the most toxic metal is smaller than 10%, while the observed mixture effect is larger than 10%.

8.3.2 Prediction performance of the mixture reference models

The CA model has been often reported to be the most conservative model of the two mixture reference models (e.g. Altenburger et al. 1996; Asselman et al. 2013; Bellas 2008; Cedergreen et al. 2008; Kortenkamp et al. 2009). Our results confirm this observation for chronic metal mixture toxicity to *C. dubia*: the CA-predicted effect was generally higher than the IA-predicted effect (Figure 8.2). Based on this observation, the CA model has been proposed as a conservative first tier in mixture risk assessment frameworks (Backhaus & Faust 2012). For a minority of treatments, IA-predicted effects where higher than CA-predicted effects, although this was only

observed when metals were combined at high effect sizes (i.e. when one of the metals individually caused at least 95% effect). However these high effect levels are of lower relevance for risk assessment. Furthermore, the difference in predicted effects between the two mixture reference models in those treatments were always smaller than 1%. Thus overall, our data confirmed that for *C. dubia* the assumption about the conservativeness of the CA model is valid.

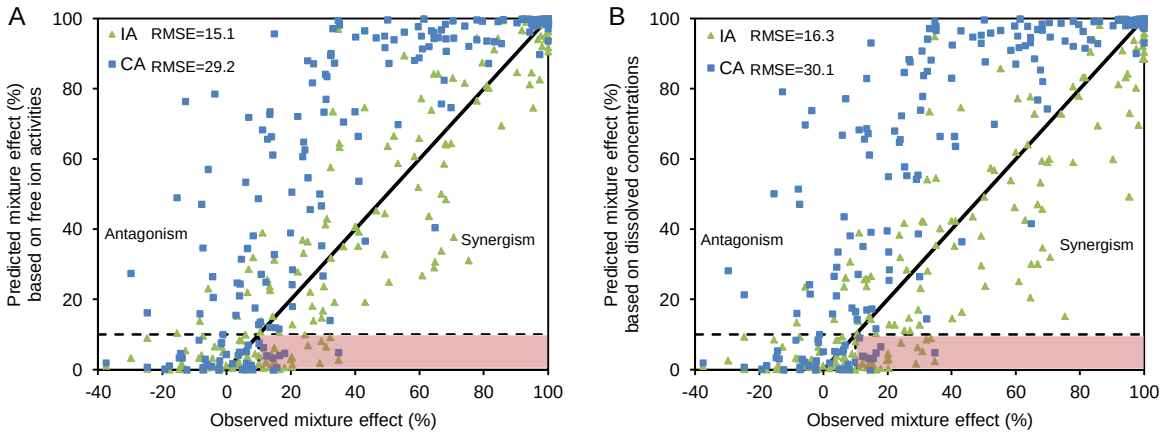

Figure 8.2. Observed mixture effect (%) versus predicted mixture effect for reproductive toxicity of Ni, Zn, Pb, Cu and Cd mixtures to *Ceriodaphnia dubia* using either the mixture reference model concentration addition (CA; squares) or independent action (IA; triangles). Predictions were based on free ion activities (A) and dissolved concentrations (B). The full line represents the perfect fit between the observed and predicted effects. Root mean square errors (RMSE) for both models are given. The red shaded area denote the situations where the predicted mixture effect is les than 10%, but the observed mixture effect is more than 10%.

Although CA was the most conservative model, the IA model was shown to be overall a better predictor of chronic metal mixture toxicity to *C. dubia* (lowest RMSE; Figure 8.2). Additionally, the mean deviation between IA-predicted and observed effects was not significantly different from 0 (one-sample t-test: p_{Me2+}=0.55 and p_{Mediss}=0.10), while this deviation of the CA model was significantly different from 0 (one-sample t-test: p_{Mediss} and p_{Me2+} <0.001, Figure 8.3). This observation was not unexpected, since most metal mixtures were shown to act antagonistically relative to the CA model, while being non-interactive relative to the IA model (Chapter 5-6, Nys et al. 2015). The CA and IA mixture reference models fundamentally differ in the assumption on how mixture constituents exert their effect on the organism in question. While the CA model assumes that mixture constituents have the same mode of action, the IA model assumes that

the constituents have different modes of action (Kortenkamp & Altenburger 2011, Jonker et al. 2005). In Chapter 5, the adherence of binary and ternary Ni, Zn, and Pb mixture toxicity to the IA model rather than the CA model was related to the presumed different modes of action of these three metals based on their effects on major ion homeostasis. However, there is still an ongoing debate whether these assumptions about similar or dissimilar mode of action are generally valid to select one of both mixture reference models as assessment model (Cedergreen et al. 2008; Kortenkamp & Altenburger 2011). Furthermore, although we have a basic understanding of the effect of metal toxicity on daphnid homeostasis for most metals considered in this meta-analysis (e.g. Pane et al. 2003; Muyssen et al. 2006), we do not yet fully understand the mode of action of these metals.

Overall, the observed patterns did not depend on whether the exposure was expressed based on free Me^{2+} activities or dissolved metal concentrations. This suggests that the influence of competition between the metals at the DOC binding sites was of minor importance in determining metal mixture toxicity in this dataset.

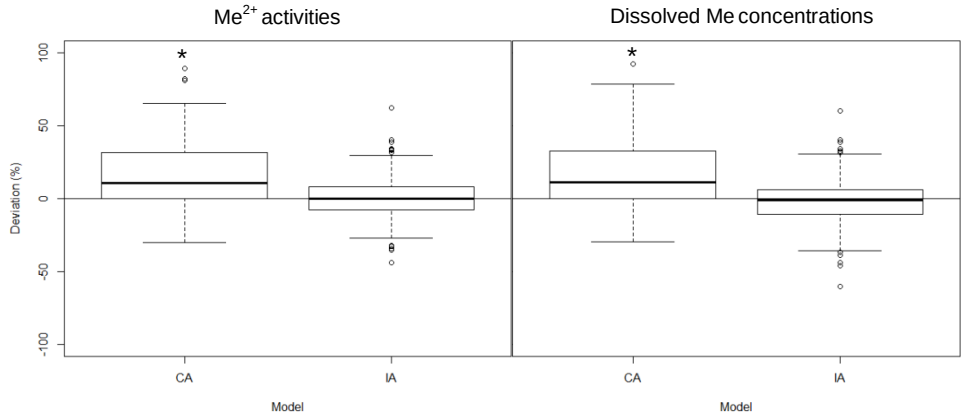

Figure 8.3. Deviation (%) between predicted and observed effects on reproductive toxicity of Ni, Zn, Pb, Cu and Cd mixtures for *Ceriodaphnia dubia.* Predictions were made using the concentration addition (CA) and independent action (IA) model based on free metal activities (left panel) and dissolved metal concentrations (right panel). Median values are given in bold, bottom and top of the boxplots give the 25th and 75th percentile. Bottom and top of whiskers represent the 5th and 95th percentile. Circles are outliers. Positive values indicate a trend towards antagonistic deviations, negative values towards synergistic deviation. Asterisks indicate that the mean deviation was significantly different from zero (one-sample t-test).

Both the IA and CA model assume that the mixture constituents do not interact. However, treatment specific deviations from non-interactivity occurred for both reference models (Table 8.2, Figure 8.4). Two meta-analyses, evaluating metal mixture effects during mainly acute exposures, have reported that the assumption of non-interactivity is often violated. Moreover, in these studies it was observed that non-interactive effects, i.e. antagonisms and synergisms, prevail over non-interactive effects in metal mixture studies (Norwood et al. 2003; Vijver et al. 2011). We observed that the abundance of non-interactive effects is dependent on the considered reference model (Table 8.2; Fisher exact test: $p<0.001$ both when the analysis was based on free ion activities and dissolved concentrations). For the IA model, the majority of treatments were non-interactive, while for the CA model antagonistic effects were as common as non-interactive effects. Synergistic deviations, which are of main concern in risk assessments, were observed only in a minority of treatments for both models, i.e. 9% for IA and 1% for CA. This suggests that the assumption of non-interactivity would be conservative in the majority of mixture situations. Similar conclusions have been made for pesticide mixtures (Cedergreen 2014) and acute metal mixtures (Vijver et al. 2011; Cedergreen 2014). For *D. magna* the occurrence of synergistic effects in binary Ni-Zn mixtures was effect size dependent (Chapter 4). However, a similar effect size dependency was not observed for the *C. dubia* mixture data (Figure 8.5).

Table 8.2. Distribution (percentafe of total values, n=210) of non-interactive and interactive (synergistic or antagonistic) treatments-specific effects relative to the independent action (IA) or concentration addition (CA) mixture reference model in the mixture treatments of the experiments investigating reproductive toxicity to *Ceriodaphnia dubia*.

	Free ion activities		Dissolved concentrations	
	IA	CA	IA	CA
Non-interactive	62	41	60	39
Antagonistic	12	40	11	42
Synergistic	9	1	12	2
Undefined[b]	17	17	17	17

[a] Deviations from non-interaction were defined as a predicted mixture effect that fell out of the 95% confidence interval of the observed mixture effect.
[b] If the observed mixture effect was 100%, no interactive effect could be assigned to the treatment.

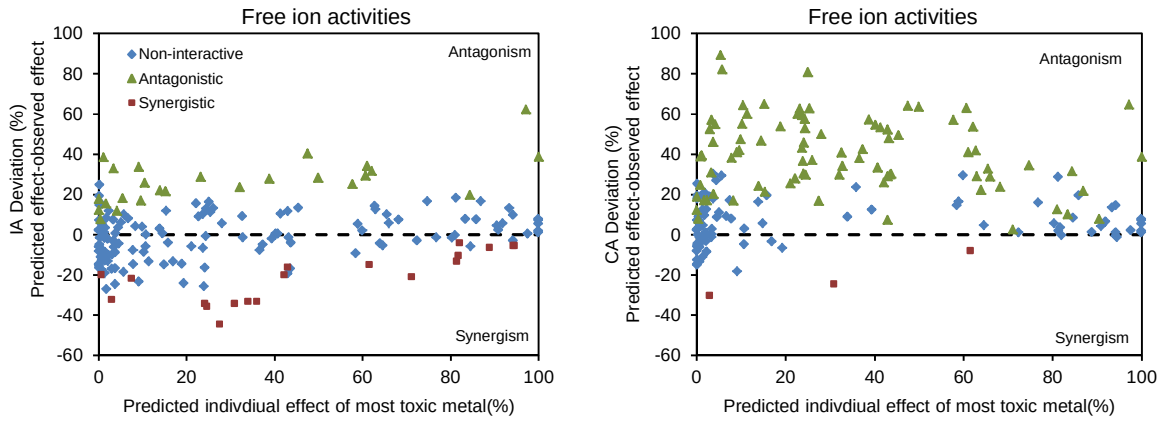

Figure 8.4. Deviation (%), expressed as the difference between predicted independent action (IA; A) or concentration addition (CA; B) effect and observed effect on reproductive toxicity of in Ni, Zn, Pb, Cu and Cd mixtures to *Ceriodaphnia dubia* as a function of the predicted individual effect (%) of the most toxic metal in the mixture. Deviations higher than one indicate possible antagonistic interactions; deviations lower than one denote possible synergistic deviations. Mixture effects were predicted based on free Me^{2+} activities. Symbols are denoted as follows: significant antagonistic interactions (triangles), significant synergistic interactions (squares), and non-interaction (diamonds). Values higher than 0 indicate a trend towards antagonistic interactions, values lower than 0 indicate a trend towards synergistic interactions.

8.3.3 Protectiveness of CA at low effect sizes

For risk assessment frameworks, mainly low concentrations are of importance, e.g. the EC10 level in the EU (EC 2003; 2011; ECHA 2008). Therefore, it is of vital importance that the models used in risk assessment frameworks are protective at these low effect levels. Figure 8.2 shows that for 82% of the mixture treatments where CA predicted maximum 10% mixture effect, observed mixture effects were indeed lower than 10%. A similar picture is given by Figure 8.5: in most treatments were $\sum TU_{EC10}$ was lower than 1 less than 10% mixture effect was observed. This suggests that also at the low effect sizes of importance for risk assessment the CA model is generally protective for metal mixture toxicity to *C. dubia*.

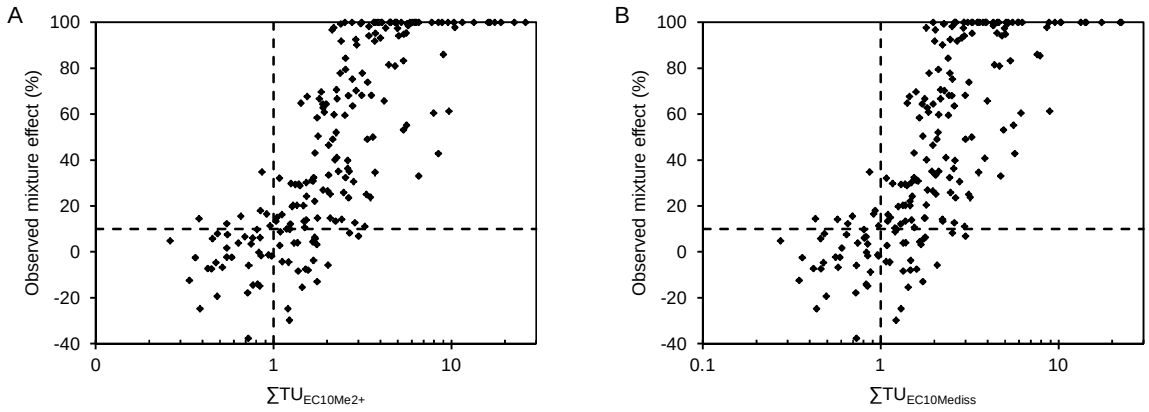

Figure 8.5. Observed mixture effects on reproductive toxicity to *Ceriodaphnia dubia* as a function of sum of toxic units, expressed relative to EC10 based on free ion activities ($\sum TU_{EC10Me2+}$; A) or dissolved concentrations ($TU_{EC10Mediss}$; B). Points at the left side of the vertical dashed line are mixture treatments where $\sum TU_{EC10Me2+}<1$, points at the right side of this line are mixture treatments where $\sum TU_{EC10Me2+}>1$. The horizontal dashed line denotes an observed mixture effect of 10%.

To investigate this matter in more detail, we calculated $EC10_{TUEC10}$ for all mixture experiments. In Figure 8.6, the cummulative distrubution of the $EC10_{TUEC10}$ in the different metal mixture experiments is shown. If the mixture effect at the EC10 follows the CA model, the $EC10_{TUEC10}$ should be equal to 1. An ECx_{TUECX} smaller than one indicates a trend towards synergistic interactions relative to the CA model, while values higher than one suggest a trend towards antagonistic interactions. The majority of $EC10_{TUEC10}$ differed less than a factor of 2 from 1. For acute pesticides mixtures (Belden et al. 2007; Cedergreen 2014) and acute metal mixtures (Cedergreen 2014) similar observations based on EC50 or LC50 (50% lethal concentrations) have been reported.

Although a few $EC10_{TUEC10}$ were lower than one, the majority of $EC10_{TUEC10}$ were higher than one. The log-normal distribution was the best fitting ditribution model out of 6 different distributions, and this both when $EC10_{TUEC10}$ were expressed based on free ion activities or dissolved concentrations. The median $EC10_{TUEC10}$, calculated as the 50% percentile of the log-normal distribution, were 1.62 and 1.54 when TU were expressed based on free ion activities or on dissolved concentrations, respectively. This suggests, that in general the CA model is conservative for chronic metal mixture toxicity to *C. dubia*.

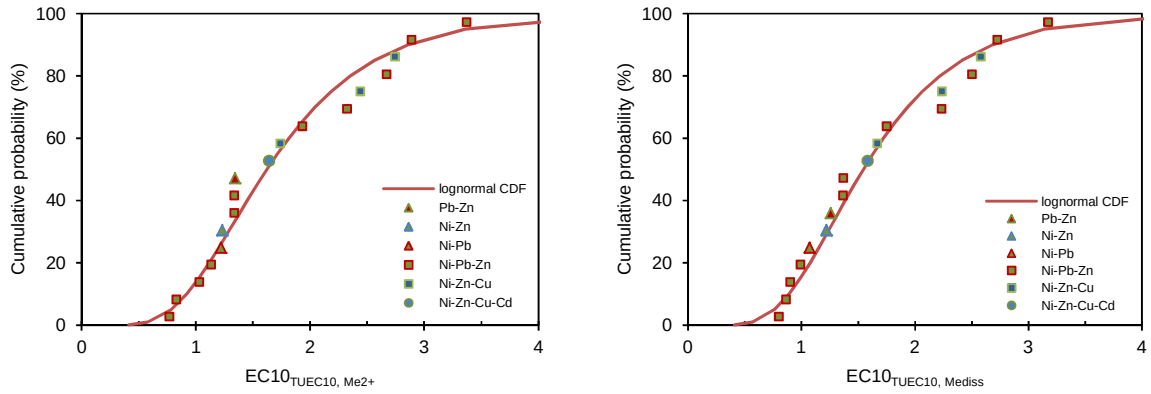

Figure 8.6. Cumulative distribution of $EC10_{TUEC10}$ for free ion activities (A) and dissolved metal concentrations (B) in the chronic metal mixture experiments with *Ceriodaphnia dubia* (reproductive toxicity). EC10 were calculated using the 2 parameter log-logistic concentration response model (Equation 8.2) .The fitted curve is the log-normal cummulative frequency distribution. Data points are plotted at the Hazen plotting position.

The same approach was used to calculate $EC10_{TUEC10}$ for two species: the crustacean *D. magna* and the algae *P. subcapitata*. The cumulative distribution of the geometric mean $EC10_{TUEC10}$ for *D. magna, P. subcapitata* and *C. dubia* is plotted in Figure 8.7. For the three considered species, the geometric mean $EC10_{TUEC10}$ ranged between 1.39-1.62 and 1.19-1.54 for free ion activities and dissolved concentrations, respectively (Figure 8.7; Table 8.3). This confirms the observation made for *C. dubia* that the CA model is protective, overall even a bit overprotective, for metal mixture toxicity to these species at low effect sizes. It would be informative to include chronic mixture toxicity data of other species and extra metal mixture combination to this species mixture toxicity distribution, to evaluate the general protectiveness of the CA model on chronic metal mixture toxicity at low effect sizes across species.

Table 8.3. Geometric mean $EC10_{TUEC10}$ of chronic metal mixture toxicity for *Ceriodaphnia dubia, Daphnia magna* and *Pseudokirchneriella subcapitata.* Standard deviations on geometric means are also reported.

	Geometric mean $EC10_{TUEC10Me2+}$	Geometric mean $EC10_{TUEC10Mediss}$
C. dubia	1.62±0.14	1.54±0.25
D. magna	1.39±0.01	1.36±0.02
P. subcapitata	1.43±0.10	1.18±0.08
Median	1.43	1.36

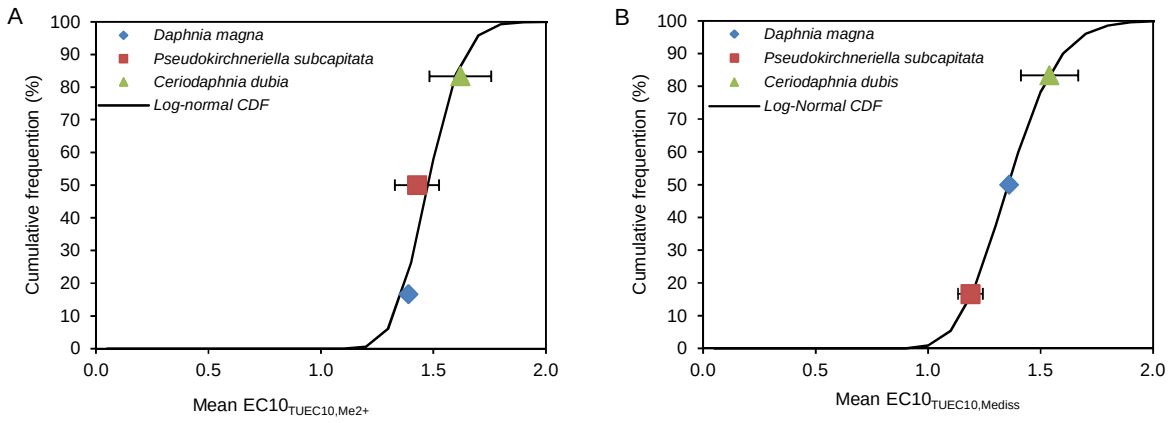

Figure 8.7. Cumulative species mixture toxicity distribution combining the geometric mean species-specific TU_{EC10} for *Daphnia magna, Pseudokirchneriella subcapitata* and *Ceriodaphnia dubia.* $EC10_{TUEC10}$ were expressed based on either free ion activities (A) or dissolved metal concentrations. The full line represents the normal cumulative distribution function. Error bars denote standard deviations on geometric means. If no error bars are shown, standard deviations were lower than 0.02 TU_{EC10}.

8.3.4 Tiered metal mixture risk evaluation scheme

In the present study, we observed that for *C. dubia* chronic metal mixture effects at low effect concentrations may be important. This suggests that protecting aquatic communities on a metal-by-metal basis, which is the current practice in metal risk assessment frameworks in the EU, could lead to an underestimation of the effects of metal mixture exposure in the environment. Consequently, a question of major importance is how we can incorporate these mixture toxicity effects in ecological risk assessment frameworks. Recently, Backhaus & Faust (2012) presented a conceptual chemical mixture risk assessment frameworks. In this framework, the CA model was suggested as a first conservative tier, while IA based methods were suggested in higher evaluation tiers.

Alternatively, Van Regenmortel et al. (2014; 2015) suggested a possible tiered metal mixture risk assessment scheme using four metal mixture risk evaluations methods (CA-SSD, IA-SSD, CA-DRC, and IA-DRC; Figure 0.7) building on the bioavailability and SSD-based risk assessment approaches for individual metals (e.g., ECI 2008; Van Sprang et al. 2009; 2016; see Introduction section 0.8.3). The CA-SSD method was proposed as a first conservative evaluation tier which identifies situations where risks are unlikely to occur (Van Regenmortel et al. 2014; 2015). In the second evaluation tier, the IA-SSD method is used to identify situations where communities are

potentially at risk due to metal mixture toxicity. In a third tier, the use of more advanced methods was suggested, such as the dose response-based evaluation methods (CA-DRC or IA-DRC) and/or bioavailability based metal mixture models (e.g., BLM-type models or WHAM-F_{TOX}) (Van Regenmortel et al. 2015).

8.3.4.1 Presentation of a possible tiered metal mixture risk evaluation scheme

Here, we propose a possible tiered metal mixture risk evaluation scheme (Figure 8.8) building on the scheme proposed by Van Regenmortel et al. (2014; 2015). This scheme combines the bioavailability normalization-based risk assessment approaches of the individual metals with the CA and IA mixture reference models. We do not make an *a priori* choice of CA or IA for metal mixture risk evaluation based on the (dis)similar mode of action of the metals, because the mode of action for metals is currently not well understood. Furthermore, the mode of action of two metals may be similar for one aquatic organism, but dissimilar in another species. For example, the major mechanism of Zn and Pb toxicity in fish has been linked to the disturbance of the Ca homeostasis for Zn and Pb (Spry & Wood 1985, Hogstrand et al. 1995; Rogers et al. 2003; Rogers & Wood 2004). Alternatively, for daphnids Zn toxicity is presumed also to occur due to a disturbance of the Ca^{2+} uptake (Muyssen et al. 2006), while the mode of action for Pb is still unclear but it has been observed that the disturbance of the Ca homeostasis is not likely the primary toxicity mechanism (Mager et al. 2011a; Chapter 2). Additionally, it has been suggested that the assumptions of (dis)similarity in mode of actions of mixtures for selecting CA or IA methods in risk assessment approaches are seldom met because chemicals may interact at several different levels of the physiological organisation of aquatic organisms (Jonker et al. 2011). Instead, both mixture reference models are applied in a sequential scheme which starts with a CA-based conservative tier and subsequently applies more liberal tiers (Figure 8.8). The different tiers and the underlying assumptions will be briefly discussed below.

The possible tiered metal mixture risk assessment scheme is presented here based on measured metal concentrations and HC5 (5% hazardous concentrations, i.e. the concentration that is hazardous to 5% of the species) instead of predicted environmental concentrations (PEC) and

predicted no effect concentrations (PNEC), for instance used by Backhaus & Faust (2012), since the derivation of the latter involves arbitrarily chosen assessment factors which makes them scientifically less relevant.

Tier 0- compliance with individual metal HC5: Ecological communities may be exposed to concentrations of metals that possess already risks individually. Tier 0 applies the approaches currently used in European ecological metal risk assessments (e.g. DEPA 2008; Van Sprang et al. 2009; 2016), i.e. the measured metal concentrations in a water sample are evaluated relative to the bioavailability-normalized HC5 of each of the individual metals. If one or more metals are present at concentrations (x_{Mei}, expressed as dissolved metal concentration) above its individual HC5 ($HC5_{Mei}$), communities are considered to be potentially at risk by one or more of the individual metals. In practice, it is evaluated if the TU expressed relative to the HC5 for metal *i* ($TU_{HC5,Mei}$) is higher than 1 (Eq. 8.8).

$$TU_{HC5,Mei} = \frac{x_{Mei}}{HC5_{Mei}} > 1 \quad (8.8)$$

The assumption underlying this tier is that the HC5 is protective for 5% of the species in the community. The validity of the latter assumption can be evaluated using micro- or mesocosms experiments. For Ni, it was recently shown that the HC5 was protective for community-level effects of Ni (Hommen et al. 2016).

Tier 1.A-CA SSD: The actual evaluation of potential metal mixture risks starts in Tier 1.A using the CA-SSD method. The CA-SSD method applies the toxic unit concept directly on the bioavailabilty-normalised HC5 of the individual metals to calculate a mixture TU, expressed relative to the HC5 ($\sum TU_{HC5}$; Eq. 8.9; Calamari & Vighi 1992; Van Regenmortel et al. 2014; 2015).

$$\sum TU_{HC5} = \sum_{i=1}^{n} \frac{x_{Mei}}{HC5_{Mei}} \quad (8.9)$$

Based on the CA concept, it can be assumed that when the $\sum TU_{HC5}$ equals 1, exactly 5% of the species of a community are affected by the mixture (Van Regenmortel et al. 2014; 2015). If the criteria used in the ecological risk assessment of the individual metals (e.g. DEPA 2008; Van Sprang et al. 2009) are extrapolated to mixture risk assessment than it can be assumed that

risks are 'acceptable' if less than 5% of the species are affected by the metal mixture, i.e. $\sum TU_{HC5}<1$.

Tier 1.B-IA SSD: The IA-SSD method applies the IA model on the SSDs of the individual metals in the mixture (De Zwart & Posthuma 2005). For every metal present in the mixture, the potentially affected fraction of the community (PAF_{Mei}; i.e. the fraction of species affected by the individual metal) is calculated from the SSD of the individual metal. The $PAF_{Mix,IA-SSD}$, i.e. the potentially affected fraction by the mixture following the IA-SSD method, is calculated using Equation 8.10:

$$PAF_{Mix,IA-SSD} = 1 - \prod_{i=1}^{n}(1 - PAF_{Me_i}) \quad (8.10)$$

The assumption underlying the IA-SSD model is that, when SSDs based on EC10s are used, that as long as the species are not exposed to metal concentrations in the mixture that exceed the EC10 of the individual metals, the effect of the mixture on each of the species will also be less than 10%. Van Regenmortel et al. (2014; 2015) showed that IA-SSD is the least conservative method when CA-SSD, IA-SSD, CA-DRC, and IA-DRC were compared. In the proposed tiered metal risk assessment scheme the IA-SSD is therefore used to identify the situations were risks of metal mixture exposure are likely to occur (i.e. when $PAF_{Mix,IA-SSD}>0.05$).

Tier 2: Standard-CA DRC: In the CA-DRC method the CA model is directly applied on the bioavailability normalized toxicity data of the species in the individual metal SSDs (Backhaus & Faust 2012; Van Regenmortel et al. 2014; 2015). For each species in the SSD, a $\sum TU_{EC10}$ is calculated using Eq. 8.6. In the standard CA-DRC approach, the potentially affected fraction ($PAF_{Mix,\ standard\ CA\text{-}DRC}$) is calculated as the fraction of species with $\sum TU_{EC10}>1$. If the $PAF_{Mix,\ standard\ CA\text{-}DRC}<0.05$, risks of metal mixture exposure are unlikely to occur.

The assumption underlying the standard CA-DRC method is that at $\sum TU_{EC10}=1$ each species would experience exactly 10% mixture effect relative to a control (Van Regenmortel et al. 2014; 2015). A requirement of the CA-DRC method is that species are shared between the SSDs of the individual metals. However, Van Regenmortel et al. (2014; 2015) reported that often only a minority of the species in a SSD of an individual metal is also present in the SSD of other metals, e.g. only 5 species are shared between the SSDs of Ni, Zn, Cu, Cd, and Co.

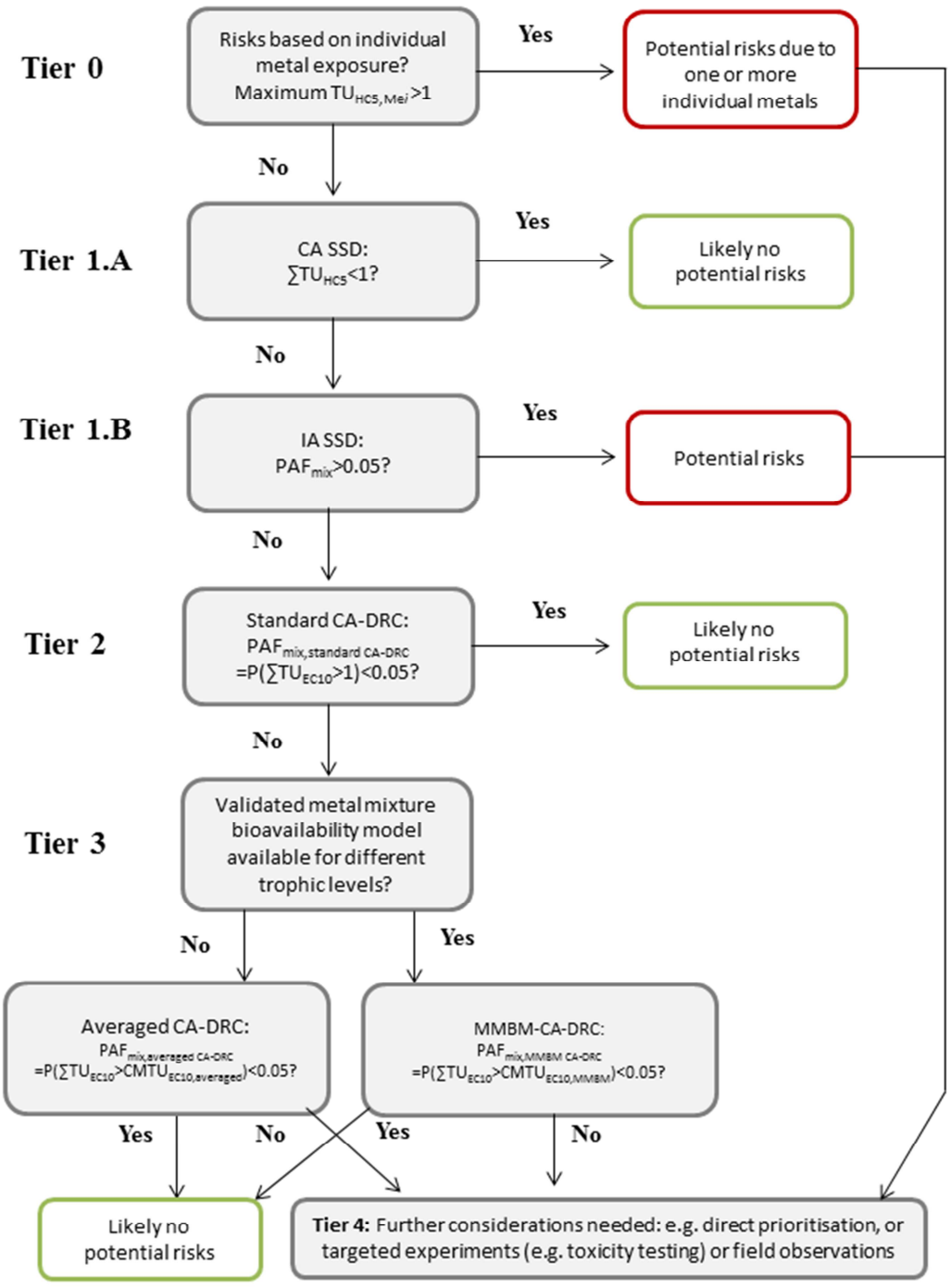

Figure 8.8. Possible tiered metal mixture risk evaluation scheme

Tier 3: Refinements of the CA-DRC approach. The assumption of the CA-DRC method that at $\sum TU_{EC10}=1$ each species would experience exactly 10% mixture effect relative to a control might be on average too conservative (Figure 8.7). Therefore, Tier 3 allows for a risk evaluation based on a refinement of the CA-DRC method which is more accurate. In Tier 3, there are two options, depending on whether metal mixture bioavailability models are available or not.

Option A: MMBM-CA-DRC method: In an ideal situation, validated MMBMs exist for all species and metal combinations. In that situation, the MMBMs can be used to predict an $EC10_{TUEC10}$ for each of the species in the SSD. These $EC10_{TUEC10}$ are dependent on the metal concentration ratio and water chemistry of the receiving water. By combining these MMBM predicted species-specific $EC10_{TUEC10}$ for all species in an SSD, a critical mixture $EC10_{TUEC10}$ can be derived. This MMBM predicted critical mixture TU_{EC10} ($CMTU_{EC10,MMBM}$) is the $\sum TU_{EC10}$ concentration which can be regarded as protective, i.e. the species would experience maximum 10% mixture effect relative to a control, for x% of the species. The percentage of species protected with this value will be a regulatory decision. The $CMTU_{EC10,MMBM}$ is depended on the metal concentration ratio and the water chemistry of the receiving water The $CMTU_{EC10,MMBM}$ is then used in the CA-DRC method to calculate a $PAF_{Mix\text{-}MMBM\text{-}CA\text{-}DRC}$.

Since this a new-concept, an example of the derivation of the 'critical mixture TU_{EC10}' for a Ni-Zn-Pb mixture for *C. dubia* using the MMBM of Chapter 7 is given in Figure 8.9. In this example, the IA MMBM is used as an example to predict a bioavailbility-normalised and metal-mixture specific $EC10_{TUEC10}$. However, for other species other types of MMBMs (e.g. CA based) might be used.

A prerequisite of the MMBM-CA-DRC method is of course that MMBMs exist for a sufficient number of species, i.e. either species-specific MMBMs exists or cross-species-validation of the MMBMs to non-model species has been proven. The European Union has set some regulations about the minimum data requirements for the use of SSDs in environmental risk assessment processes. For instance, data of at least 8 different taxonomic groups must be available (EC 2003). Additionally, there are also some minimum requirements about which taxonomical groups should be represented in the toxicity database. These requirements can from a guidance in the future development of chronic MMBMs for application in metal mixture risk assessment frameworks. To allow the use of MMBMs in risk assessment approaches, it is necessary that

future metal mixture studies focus on the further development of these chronic metal mixture bioavailability models.

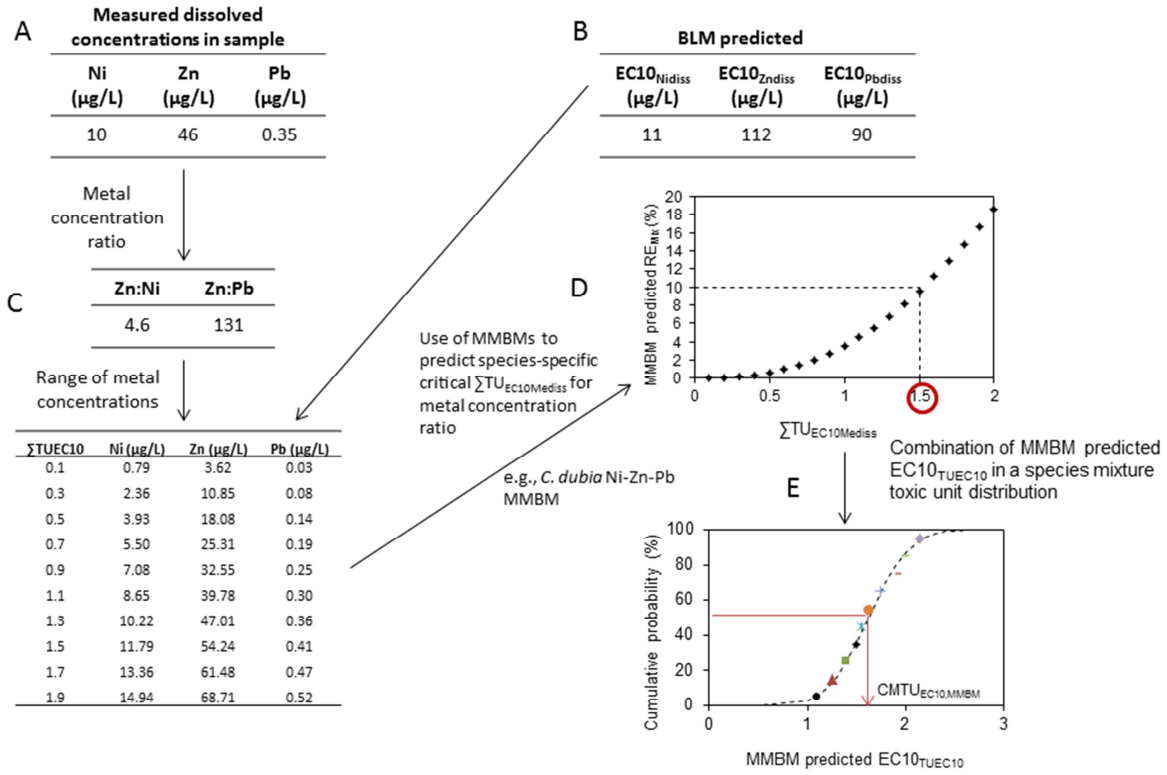

Figure 8.9. Overview of the method to derive MMBM predicted species-specific critical $\sum TU_{EC10Mediss}$ for the MMBM-CA-DRC method: A) Monitoring results provide dissolved metal concentrations and water chemistry (e.g. DOC, pH, Ca,…); B) Using the individual metal BLMs $EC10_{Mei}$ are calculated; C) Based on the metal concentration ratio in the sample a range of metal concentrations is derived for a $\sum TU_{EC10Mediss}$ range (e.g. 0.1-1.9); D) MMBMs are used to predict species-specific $EC10_{TUEC10Mediss}$ for the metal concentration ratio in the receiving water (e.g. in this example the *Ceriodaphnia dubia* Ni-Zn-Pb MMBM from Chapter 7 was used to predict a *C. dubia* $EC10_{TUEC10Mediss}$); E) All calculated species-specific $EC10_{TUEC10,Mediss}$ are combined in a species mixture TU distribution and a critical mixture TU_{EC10} for the MMBM ($CMTU_{EC10,MMBM}$) is derived. In this figure derived at the arbitrarily chosen cummulative probability of 50%. The $CMTU_{EC10,MMBM}$ is then applied in the CA-DRC method (the species mixture TU distribution shown here is a hypothetical distribution, since validated MMBMs are lacking for all species except *C. dubia*).

Option B: 'averaged'-CA-DRC method If chronic MMBMs are not available, a more ecological-relevant application of the CA-DRC method would be the 'averaged'-CA-DRC method. In the 'averaged'-CA-DRC method, a critical mixture TU_{EC10} ($CMTU_{EC10,averaged\ CA\text{-}DRC}$) is derived based on the

geometric means of the available chronic metal mixture toxicity data (expressed as TU_{EC10}) in a species mixture TU distribution. The $CMTU_{EC10,averaged\ CA\text{-}DRC}$ is then used in the CA-DRC to calculate a $PAF_{Mix\text{-}Averaged\text{-}CA\text{-}DRC}$. In Figure 8.7, a distribution of the geometric mean chronic metal mixture $EC10_{TUEC10}$ for a few species is shown. If chronic data becomes available for more species, these data can be used to calculate a more accurate critical mixture TU_{EC10}.

The difference between this 'averaged' critical mixture TU_{EC10} and the MMBM predicted critical mixture TU_{EC10} is that the latter is derived from a species distribution of bioavailability normalised species mixture $EC10_{TUEC10}$. Hence, the $CMTU_{EC10,MMBM}$ is dependent on the water chemistry, the metal mixture ratio in the receiving water and the availability of metal mixture bioavailability models. The 'averaged' critical mixture TU_{EC10}, on the other hand, is derived from a species distribution of geometric means of the observed, non-bioavailability normalized species mixture $EC10_{TUEC10}$. As such, the $CMTU_{EC10,averaged}$ is only dependent on the data availability and is independent of water chemistry and metal mixture ratio. This 'averaged' critical mixture TU_{EC10} gives an overall picture of the protectiveness of the CA mixture reference model at low effect levels.

Tier 4: Further considerations needed: If the outcome after Tier 3 is still unclear, i.e. samples for which $PAF_{MMBM\text{-}CA\text{-}DRC}$ or $PAF_{Mix\text{-}averaged\text{-}CA\text{-}DRC}$ are higher than 0.05, further actions are needed. These can be either the direct prioritization of lowering metal concentrations in these sites. Alternatively, if direct prioritization would be too costly, metal mixture toxicity risks in these sites may be further evaluated using either targeted experiments (laboratory and/or field bioassays) or based on field collected ecological data, or a combination of both (Gerhard et al. 2004; Gerhard et al. 2008). It has to be kept in mind that there are some limitations to either of these methods, e.g. toxicity testing may be costly and time-consuming, the outcomes may be variable depending on the considered test species or the sampling occasion (Ragas et al. 2011). The species included in the bioassay are important since metal sensitivities may differ greatly between aquatic organisms. Additionally, an organism might be sensitive to one metal, but rather insensitive to others. As a consequence, the organisms in a mixture bio-assay should be carefully selected.

8.3.4.2 Application of the tiered metal mixture risk evaluation scheme on a monitoring dataset

To show the use of the tiered risk evaluation scheme in identifying monitoring sites where communities are potentially affected by metal mixture exposure, we applied the tiered risk evaluation scheme on a monitoring dataset with dissolved Ni, Zn and Pb concentrations measured in 405 samples in over 80 sampling sites in the Dommel river basin in 2010. The Zn:Ni & Zn:Pb ratios in the Dommel monitoring dataset are plotted in Figure 7.1. Details on the bioavailability normalization approach are given in appendix. In short, chronic EC10 or NOECs in the toxicity databases of Ni, Zn, and Pb were normalized using the bioavailability normalization approaches used in the European risk assessment frameworks for these metals (DEPA 2008; Van Sprang et al. 2009; 2016; Nys et al. 2016). The evaluation of the tiered metal mixture risk evaluation scheme is shown in Figure 8.10.

Tier 0 compliance with individual metal HC5: More than half of the 405 samples were predicted to be potentially at risk based on the presence of one of the individual metals (Figure 8.10). When one of the metals was present above his individual HC5 this was mostly the case for Zn (i.e. $TU_{HC5Zn} \geq 1$), or for both Ni and Zn (i.e. $TU_{HC5Zn} \geq 1$ & $TU_{HC5Ni} \geq 1$). In a minority of monitoring points only Ni was present above its individual HC5 concentration, while Pb was never present in concentrations above its individual HC5.

Tier 1.A CA-SSD: For 128 monitoring samples evaluated in Tier 1.A, the $\sum TU_{HC5,Mix}$ was lower than 1, and as such no risks are expected in these sites (Figure 8.10).

Tier 1.B-IA-SSD: The $PAF_{Mix\text{-}IA\text{-}SSD}$ was higher than 0.05 for 11 of the 56 samples considered in this tier (Figure 8.10). These monitoring sites are thus considered to be potentially at risk by metal mixture exposure.

Tier 2-CA DRC: For only 3 of the 45 monitoring samples further evaluated in Tier 2, $PAF_{Mix\text{-}CA\text{-}DRC}$ was lower than 0.05 (i.e. at the $\sum TU_{EC10}=1$ cut-off; Figure 8.10). In these 3 samples, risks of metal mixture exposure are unlikely to occur.

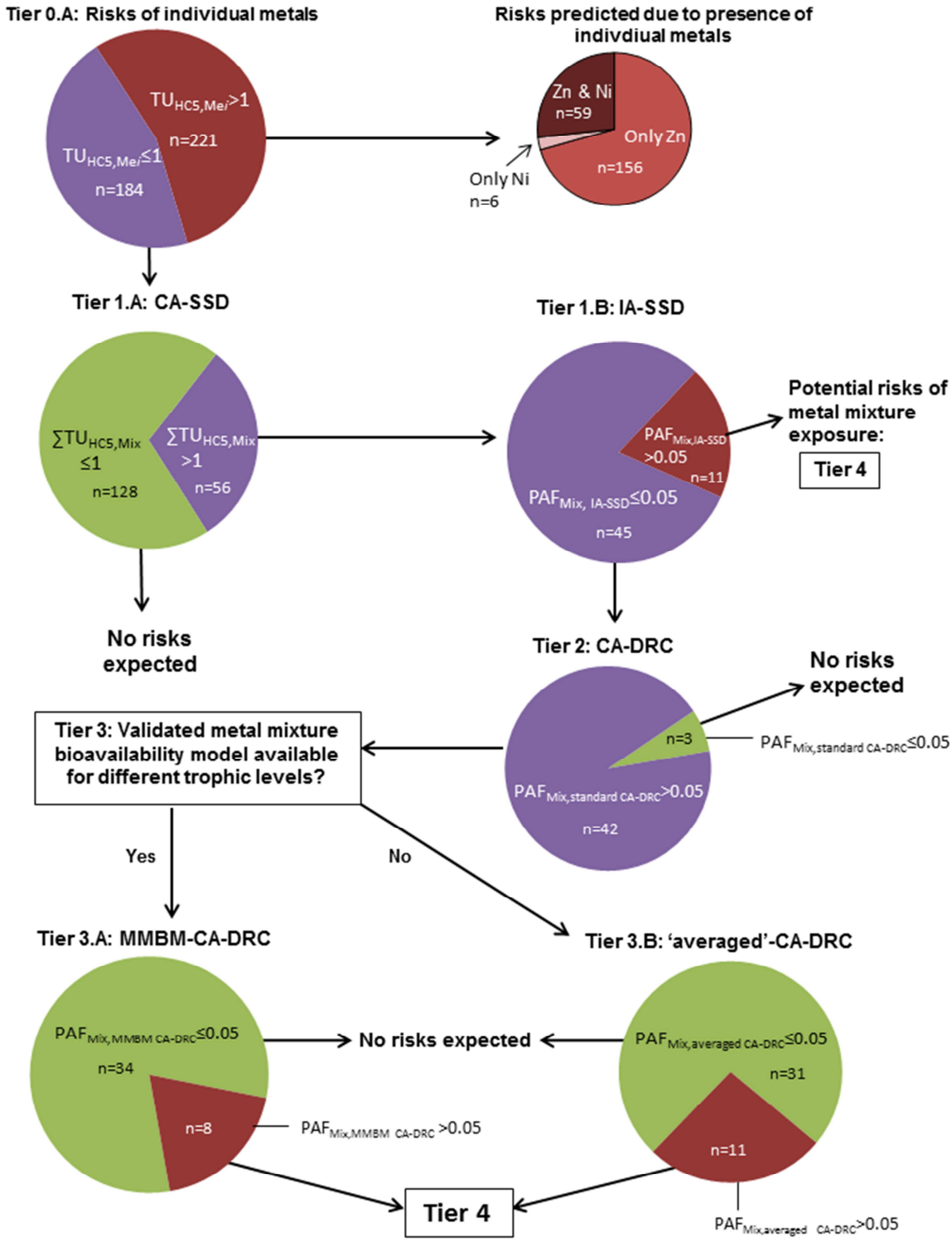

Figure 8.10. Application of Tier 0 to Tier 4 of the possible metal mixture risk evaluation scheme on the environmental monitoring data of the Dommel for Ni-Zn-Pb mixtures. Color codes are designated as follows: red: potential risks of metal (mixture) exposure; green: no risks of metal (mixture) exposure expected; purple: further consideration in a higher tier needed. An overview of the different steps in the tiered risk assessment scheme is given in Figure 8.8 and in the text.

Tier 3.A- MMBM-CA-DRC: In theory, Tier 3.A can only be applied if validated chronic MMBMs exist for all species. However, we currently lack chronic MMBMs for most species. Therefore, in the present analysis, we assumed that all species are similar to *C. dubia,* i.e. the effects of water chemistry on metal (mixture) toxicity are the same between species, mixture toxicity of Ni-Zn-Pb can be predicted using the *C. dubia* MMBM of Chapter 6. Under this, in practice, unrealistic assumption, the MMBM predicted *C. dubia* $EC10_{TUEC10}$ can be regarded as the critical mixture $EC10_{TUEC10}$. This MMBM predicted critical mixture $EC10_{TUEC10}$ (which is a function of water chemistry and metal concentration ratio) is then used as $\sum TU_{EC10}$ cut-off to calculate $PAF_{Mix,MMBM\text{-}CA\text{-}DRC}$ in the MMBM-CA-DRC method. Using this approach, $PAF_{Mix,MMBM\text{-}CA\text{-}DRC}$ in 34 samples was lower than 0.05, for these samples no risks are thus expected (Figure 8.10). Alternatively, for 8 samples $PAF_{Mix,MMBM\text{-}CA\text{-}DRC}$ was higher than 0.05, following the tiered risk assessment scheme these samples should be further considered in Tier 4.

Tier 3.B-'averaged'-CA-DRC: A preliminary species mixture toxicity distribution is shown in Figure 8.7. From this figure an 'averaged' critical mixture TU_{EC10} can be derived, for instance the median of the geometric mean species-specific $EC10_{TUEC10}$ (1.36; Table 8.3). Using this median $EC10_{TUEC10}$ as the 'averaged' critical mixture TU_{EC10} to calculate the potential affect fraction ($PAF_{Mix,averaged\text{-}CA\text{-}DRC}$), resulted in 31 samples where the $PAF_{Mix,averaged\text{-}CA\text{-}DRC}$ was lower than 0.05. For 11 samples, the $PAF_{Mix,averaged\text{-}CA\text{-}DRC}$ was higher than 0.05, following the tiered risk assessment scheme these samples should be further considered in Tier 4.

Overall, we observed using the tiered risk assessment scheme that communities in some Dommel sites might be at risk due to metal exposure. However, the majority of potential risks were predicted to occur due to the toxicity of one, or more individual metals. Risks of metal mixture exposure were identified in only a minority of the monitoring samples, i.e. 11 samples in Tier 1.B (IA-SSD) and another 8 or 11 in Tier 3 depending on the method used. Overall, this in line with the observations reported for Ni, Zn, and Cu mixtures in this tributary (Van Regenmortel et al. 2015).

8.3.4.3 General considerations of the proposed tiered risk assessment scheme

In the present study, we proposed a possible metal mixture risk evaluation scheme combining both CA and IA with species sensitivity distributions, bioavailability normalisations and metal mixture bioavailability models. The metal mixture risk evaluation scheme presented here is only one of the possible approaches. We recognise that the availability of additional chronic metal mixture toxicity data in the future will allow to further refine the proposed method. Additionally, following issues currently hinder the application of this scheme, or possible alternatives, in metal mixture risk assessment frameworks. First, several assumptions underlie the different tiers of the proposed metal mixture risk evaluation scheme. Until now, these assumptions have not yet been tested. Confirmation of the underlying assumptions and an assessment of the overall degree of conservativeness at the community-level would increase the confidence in this approach. Therefore, there is a need for studies investigating community-level metal mixture toxicity effects either based on multi-species experiments (e.g. meso- or microcosm) and/or field data. Second, the paucity of validated chronic MMBMs currently limits the application of Tier 3.A. Consequently, there is need for validated chronic MMBMs for different species and metal combinations. Third, the application of the tiered metal mixture risk assessment was evaluated based on measured dissolved concentrations, while it is well known that metals may compete with each other for the binding sites of DOC (e.g. see Chapter 5). A risk assessment based on dissolved concentrations might lead to some overestimation of metal mixture risks. Hence, metal mixture risks should ideally be evaluated on the free ion activity level. However, an evaluation based on free ion activities is currently limited due to the use of different speciation programs in the different risk assessment processes of the individual metals, e.g. WHAM V for Zn (Van Sprang et al. 2009), WHAM VI for Ni (DEPA 2008) and Visual Minteq for Pb (Van Sprang et al. 2006). An uniformisation of the speciation calculations performed in these methods would allow to account for the competing effects between metals at the binding sites of DOC. In Chapter 2, we showed that recalibrating the daphnid bioavailability models of Ni, Zn, and Pb in WHAM VII resulted in similar predictive capacities as the original bioavailability models. Confirmation of this observation for the other bioavailability models (also for other species) used in the risk assessment approaches for metals would allow a metal mixture risk assessment based on free ion activities. Resolving these issues would increase the confidence in the proposed approach.

9

General conclusions & future research recommendations

9. General conclusion and future research recomendations

9.1 Introduction

At the start of the present study, the incorporation of metal mixture toxicity in risk assessment frameworks was hindered due to absence of clear patterns emerging from metal mixture studies and the paucity of chronic metal mixture studies (Van Genderen et al. 2015; Meyer et al. 2015a). The aim of the present study was to increase the understanding of chronic mixture effects in Ni, Zn, and Pb mixtures and to evaluate the implications of metal mixture toxicity for risk assessment frameworks. To address these issues, we investigated chronic metal mixture toxicity and bioavailability to daphnids. The focus was both on describing the interactive effects occurring in metal mixtures using commonly applied mixture reference models as well as on modelling metal (mixture) toxicity using a bioavailability based approach.

In the present chapter, we summarize the main conclusions of each chapter of this dissertation and relate these back to the original research question. Additionally, the links between the different chapters are emphasized and future research needs are identified. Where applicable applications of the results in risk assessment frameworks are addressed.

9.2 Development and validation of a biotic ligand model for predicting chronic toxicity of lead (Pb) to *Ceriodaphnia dubia*

The main research questions in Chapter 2 were I) what are the individual effects of Ca and pH on chronic Pb toxicity to *Ceriodaphnia dubia*?; II) can chronic Pb toxicity to *C. dubia* be predicted using a biotic ligand model (BLM)?

We observed that Ca did not significantly influence chronic Pb toxicity to *C. dubia,* whereas a high pH (8.2) provided protection against Pb toxicity (compared to lower pH levels). Based on these result a BLM was developed. In this BLM the effect of pH was modeled as a single biotic ligand site competition effect and no other competition constants were needed. The developed BLM was shown to predict chronic Pb toxicity to *C. dubia* with reasonable accuracy in an independent validation with three other datasets. Our results suggest that bioavailability based

risk assessment or water quality criteria for Pb are likely to be more appropriate than a simple hardness-based assessments or criteria.

Meanwhile, it has been shown that the chronic *C. dubia* Pb BLM can be used to predict Pb toxicity to other invertebrates such as the rotifer *Brachionus calyciflorus* and the snail *Lymnaea stagnalis* (Van Sprang et al. 2016). Moreover, the developed *C. dubia* Pb BLM has recently been integrated in a bioavailability-based effects assessment for lead (EC 2013b; Van Sprang et al. 2016). This Pb effects assessment approach has also been used to calculate bioavailability normalized HC5s for Pb in Chapter 8.

Suggestions for further research

There are still some uncertainties about the individual effects of Ca on chronic Pb toxicity to *C. dubia*. Chronic Pb toxicity was slightly higher at low Ca concentrations compared to higher Ca concentrations, although the linear relationship between Ca^{2+} and Pb^{2+} toxicity, expressed as $EC50_{Pb2+}$, was not significant. Such an effect can either be explained as a competitive effect of Ca at the Pb biotic ligand site or as a result of the combination of both low Ca stress and Pb stress. An evaluation of the individual effect of Ca on chronic Pb toxicity at a range of low Ca concentrations combined with an analysis of the uptake patterns of Pb^{2+} and Ca^{2+} might shed further light on this issue.

The overall evidence (based on data in Chapter 2 and Mager et al. (2011a)) suggests that disturbance of the Ca homeostasis is not the primary mechanisms of Pb toxicity. Until now, alternative mechanisms have still not been identified, although it has been suggested that uptake of Pb^{2+} occurs by mimicry of a different ion through a channel or transporter with low Ca^{2+} affinity and high Pb^{2+} affinity (Mager et al. 2011b). Therefore, in order to understand mechanisms of chronic Pb toxicity to *C. dubia* more clearly it would be of interest to investigate this matter into more detail.

9.3 Calibration of the chronic daphnid Ni, Zn, and Pb bioavailability models in WHAM VII

In Chapter 3, the research questions were: I) can the existing chronic daphnid bioavailability models for Ni, Zn, and Pb be updated to WHAM VII?; II) does the identity of the thermodynamic database for inorganic complexation influence the model predictions with WHAM VII, i.e. is there a difference in model predictions when the NIST updated stability constants for inorganic complexation are used compared to the default constants in WHAM VII?

The chronic daphnid bioavailability models for Zn, Ni and Pb, originally developed in WHAM V, WHAM VI, and Visual Minteq (Heijerick et al. 2005; Deleebeeck et al. 2008; Chapter 2), respectively, could be used in combination with WHAM VII to accurately predict chronic Zn, Ni and Pb toxicity to daphnids without recalibrating the actual biotic ligand binding constants. Moreover, the predictive performance in WHAM VII approached those of the original bioavailability models. With respect to the second research question, we observed that the inorganic stability constants reported by NIST described chronic metal toxicity more accurately than the default WHAM VII inorganic stability constants, especially for Ni and Zn.

These observations suggest that the general bioavailability based European risk assessment approaches for metals, which are currently based on various speciation programs and DOC assumptions, may all be updated to WHAM VII with a common DOC assumption. Such a uniformisation of the risk assessment approaches would allow a more straight forward risk assessment for metals in general and more specifically for metal mixtures. However, it remains to be tested if this conclusion is generally applicable for all chronic metal bioavailability models.

<u>Suggestions for further research</u>

As mentioned above, metal (mixture) risk assessment approaches would benefit from the uniformisation of speciation calculations. Therefore, it is necessary to evaluate if all chronic bioavailability models used in metal risk assessment approaches can be updated to WHAM VII.

It has been reported that the default stability constants for organic complexation in the WHAM VI speciation software underestimates Ni-fulvic acid binding (De Schamphelaere et al. 2006; Van Laer et al. 2006). We observed that Ni toxicity predictions in WHAM VII were slightly less well predicted than when the models where combined in WHAM VI (De Schamphelaere et al. 2006;

Deleebeeck et al. 2008), which might potentially be explained by an underestimation of the Ni-fulvic acid binding in WHAM VII. It would be interesting to evaluate the influence of WHAM VII Ni-fulvic acid complexation parameters on the predictions of Ni^{2+} activities in natural waters.

9.4 Cross-species-validation of the chronic Zn *D. magna* BLM for *C. dubia*

In chapter 4, we had one research question: Can chronic Zn toxicity to *C. dubia* be predicted using the existing chronic Zn *D. magna* BLM (Heijerick et al. 2005)?

Overall, *C. dubia* was more sensitive to chronic Zn toxicity than *D. magna*. The chronic Zn *D. magna* BLM (Heijerick et al. 2005) did not accurately predict Zn toxicity to *C. dubia,* even if the model was specifically calibrated on the sensitivity of *C. dubia*. This could be explained based on the effect of pH on Zn^{2+} toxicity, which seems stronger for *C. dubia* compared to *D. magna*. A preliminary *C. dubia* specific chronic Zn bioavailability model was developed using a log-linear pH effect instead of the single biotic ligand-site H^+ competition effect. Additionally, it was assumed that the competition effects of Ca^{2+}, Mg^{2+} and Na^+ at the Zn biotic ligand site are the same for *C. dubia* and *D. magna*. The model was shown to predict chronic Zn toxicity to *C. dubia* within 2-fold error in natural waters in the pH range from pH 7 to 8.3. However, Zn toxicity was considerably underestimated at lower pH. As a consequence, the application of the model for pH below 7 should at the moment be avoided. Based on our study, the assumption that the competitive effects between metals and other cations, such as Mg^{2+}, Ca^{2+}, H^+ and Na^+ for binding at the biotic ligand are similar between invertebrates and only metal sensitivities vary between invertebrate species commonly applied in metal risk assessment frameworks (Van Sprang et al. 2009; 2016; Schlekat et al. 2010), might be too simple in reality.

Suggestions for further research

To be able to apply the developed *C. dubia* bioavailability model in a broader pH range, the model should be validated in the lower pH range, which is representative for some European surface waters. According to the database of FOREGS (2005), the lower 10^{th} percentile for pH in European surface waters is 6.4. Hence, a validation in the pH range 6.4 to 7 seems appropriate.

The difference of the effect of pH on chronic Ni (De Schamphelaere et al. 2006) and Zn (Chapter 4) toxicity between the closely related species, *D. magna* and *C. dubia*, deserves further attention, since these are considered as standard model organisms in metal risk assessment frameworks (DEPA 2008; Van Sprang et al. 2009; 2016; Schlekat et al. 2010). Furthermore, the uncertainty associated with the selection of either the chronic Zn *D. magna* BLM or the chronic Zn *C. dubia* bioavailability model for normalizing chronic invertebrate toxicity data in Zn risk assessment processes should be assessed.

9.5 Reproductive toxicity of a Ni and Zn mixture to *D. magna*

In Chapter 5, we investigated the chronic toxicity of the binary Ni-Zn mixture to *D. magna*. In this chapter, the main research questions were I) do Ni and Zn interact on chronic toxicity to *D. magna* or not (evaluated relative to the two commonly applied mixture reference models), II) is there a difference in observed interactive effects when the metals are combined at low or high effect sizes?

The analyses showed that the Ni and Zn mixture produced globally (i.e., analyzed based on all mixture data) noninteractive effects on reproductive toxicity according to the concentration addition model and synergistic effects according to the independent action model. However, for both mixture reference models the type of interactive effects was highly effect size–dependent. When Ni and Zn were combined at concentrations that in themselves caused a <20% effect on reproduction, only noninteractive or small antagonistic mixture effects were observed. However, when both metals were present in the mixture above a certain threshold of toxicity (i.e., where both metals already caused a >20% (for independent action) and a >40% (for concentration addition) effect on reproduction on their own), synergistic effects relative to both reference models were observed. Overall, very similar patterns of mixture toxicity were observed in the 2 independent data sets, which suggests that in contrary to what has been previously reported (Cedergreen et al. 2007) concentration-dependent interactive effects were reproducible.

Based on the results, both the concentration addition and independent action can serve as a protective scenario for *D. magna* reproductive toxicity to mixtures of Ni and Zn because mainly low effect sizes are of importance in most regulatory frameworks. In addition, the results point

out that interactive effects at low effect sizes may be different from those at high effect sizes. Therefore, extrapolations of metal mixture interactions from high to low effects sizes should be handled with care.

Suggestions for further research

The knowledge about interactive effects of metals on the physiological level, might increase our understanding of metal mixture toxicity effects. However, the interactive effects on *D. magna* toxicity observed in the present study could not be explained based on previously reported competitive effects between Ni^{2+} and Zn^{2+} for uptake by *D. magna* during short-term exposure to metal mixtures (Komjarova et al. 2008). However, differences in exposure time (chronic vs short time; Cooper at al. 2009) and exposed life stage (juveniles vs adult daphnids; Zhu et al. 2011) between our study and the one of Komjarova et al. (2008) could confound this comparison. Alternatively, we suggested that a metal-induced regulation of metal transferring proteins may have resulted in the observed synergistic effect on toxicity to the binary Ni–Zn mixture. In general, it can be concluded that there is a need to investigate the Ni-Zn mixture toxicity mechanistically, e.g., on the molecular and physiological levels.

9.6 Reproductive toxicity of binary and ternary mixture combinations of Ni, Zn, and Pb to *C. dubia*

The main research question in Chapter 6 was: How do Ni, Zn, and Pb interact on reproductive toxicity to *C. dubia* in all possible binary and ternary mixture combinations of Ni, Zn, and Pb? More specifically, we investigated if the toxicity of their binary and ternary mixtures follows the IA model rather than the CA model, given the suspected different modes of action of these metals. Additional questions were I) are interactive effects different between closely related species?; II) does the expression of exposure influence the observed interactive effect?

The toxicity of the metals in most mixtures, either expressed as free metal ion activities or as dissolved metal concentrations, were antagonistic relative to the concentration addition (CA) model, whereas no significant ($p<0.05$) interactive effects were observed relative to the independent action (IA) model. The only exception was the binary Pb-Zn mixture, for which mixture effects were non-interactive based on the dissolved concentrations, but antagonistic

based on free ion activities all relative to the IA model. The latter could be attributed to chemical speciation effects. Overall, the IA model fitted the observed toxicity better than the CA model, which is consistent with the different modes of action of these metals.

The data suggested that the joint effect of Ni-Zn mixtures on reproductive toxicity are different between *D. magna* and *C. dubia*. These differences in type of interactive mixture effects between *C. dubia* and *D. magna* warns against the extrapolation of interactive effects between species, even when they are closely related.

Both the CA model as well as the IA model provided conservative predictions for Ni, Zn, and Pb mixture toxicity, since no significant synergistic interactions were observed for any of the mixtures. However, the use of the CA approach will potentially result in an overestimation of the effects of Ni, Zn, and Pb on *C. dubia* reproductive toxicity

Suggestions for further research

As already explained above, despite the considerable research available, it is clear that we do not yet fully understand how metals interact in mixtures. This is partly due to the fact that mechanistic studies are still largely lacking (but see e.g. Komjarova et al. 2008). Investigations into the mechanistic basis of these mixture interactions (e.g. at the bioaccumulation level) may lead to a better understanding of the observed interactive effects as well as the differences in observed interactive mixture effects between species.

9.7 Development of a metal mixture bioavailability model to predict chronic Zn-Ni-Pb mixture toxicity to *Ceriodaphnia dubia*

In Chapter 7, the main research question was whether chronic Ni-Zn-Pb mixture toxicity to *C. dubia* could be predicted using a chronic metal mixture bioavailability model (MMBM). Other questions where: I) does the observed interactive effect depends on the metal concentration ratio considered, i.e. are there differences in interactive effects between *equitoxic* metal mixture rays and *environmentally* realistic mixture rays. II) what are the effects of water chemistry on chronic Ni-Zn-Pb mixture toxicity?

The mixture effects in the *equitoxic* rays were antagonistic relative to the concentration addition model, while they were mostly non-interactive relative to the independent action model, when analysed based on the free metal ion activities. In contrast, for both reference models mixture effects in the *environmental* rays were always non-interactive, when analysed on free metal ion activities. This suggests that observations of metal mixture toxicity using equitoxic metal concentration ratios, which are often used in metal mixture experiments, are probably not always representative for realistic mixture scenarios. The mixture effects shifted from non-interactivity to antagonism with increasing pH, but only when evaluated relative to the independent action model and only in the equitoxic mixture ray. Thus, water chemistry determined the type of interactive effect observed to some extent.

The developed chronic metal mixture bioavailability model (MMBM) combined the chronic *C. dubia* bioavailability models for Pb (Chapter 2), Zn (Chapter 4) and Ni (De Schamphelaere et al. 2006) with the independent action mixture reference model. The MMBM assumes that each metals binds to its own biotic ligand (BL) site and that metal (mixture) toxicity is related to the concentration of free metal ion binding to its metal-specific BL. Additionally, based on the results from Chapter 6, it is assumed that Ni^{2+}, Zn^{2+} and Pb^{2+} do not compete for binding at the biotic ligand sites. The MMBM predicted chronic toxicity of the ternary Ni-Zn-Pb mixture in natural waters differing in pH, Ca, and/or dissolved organic carbon at least equally accurately as the observed toxicity in the individual metal treatments. Therefore, chronic MMBMs are a promising tool to predict chronic metal mixture toxicity under varying water chemistry and could be potentially used in metal risk assessment frameworks (see Chapter 8).

Suggestions for further research

The developed MMBM was validated in 6 (modified) natural waters. Water chemistry variables across the waters (pH range: 7-8; Ca range: 1-2 mM; DOC range: 5-12 mg/L) covered a significant part of the ranges typically found in European surface waters, i.e. 10-90th percentiles for pH: 6.4–8.3; DOC: 0.9-17.0 mg/L; and Ca: 0.01–2.98 mmol/L (FOREGS 2005). Broadening of the validation ranges of the *C. dubia* Ni-Zn-Pb MMBM would futher increase the confidence in the application of the MMBM in future metal mixture risk assessment frameworks.

Before chronic MMBMs can be incorporated in future metal mixture risk assessment frameworks, there is a need to develop additional chronic metal mixture bioavailability models. More

specifically, the applicability of this type of model for other aquatic organisms as well as additional metal combinations has to be evaluated. ECHA (2008) has described some general rules for the requirements regarding the use of chronic metal bioavailability models in the current European risk assessment processes (i.e. the metal-by-metal approaches). For instance, bioavailability correction can only be conducted if bioavailability models are available for the following three taxonomic levels: invertebrates, algae and fish (ECHA 2008). Additionally, in order to use a 'full bioavailability correction' a 'spot-checking' of the applicability of chronic bioavailability models for non-model species is required (ECHA 2008, see Schlekat et al. 2010; Van Sprang et al. 2016 for examples for individual metals). These requirements can be a guideline in the development and cross-species validation of chronic MMBMs.

9.8 General conclusions about interactive effects of chronic Ni, Zn, and Pb mixture toxicity to daphnids

In conclusion, the interactive effects on chronic reproductive toxicity of Ni, Zn, and Pb mixtures to daphnids are dependent on the considered mixture reference model, the test organism, the applied metal concentration ratio, the concentrations of the individual metals and the metal combination. Furthermore, metals compete for DOC binding sites and as such also the expression of exposure (dissolved concentrations vs. free ion activities) determines the observed interactive effects. Hence, chronic metal mixture toxicity to daphnids is as variable as the metal mixture effects reported for acute exposures (reviewed by Norwood et al. 2003; Vijver et al. 2011)

Additionally, water chemistry variables, e.g. pH, may influence the interactive effects. However, chronic Ni-Zn-Pb mixture toxicity was shown to be predicted with reasonable accuracy using a chronic metal mixture bioavailability that combined the individual chronic *C. dubia* bioavailability models for Pb, Zn and Ni with the independent action mixture reference model.

9.9 Chronic metal mixture toxicity to *C. dubia*: implications for metal mixture risk assessments

In Chapter 8, all mixture toxicity data for *C. dubia* were combined in a meta-analysis to answer the following three risk assessment related questions: I) are mixture effects important?; II) is the CA model a conservative model for mixture toxicity, certainly at the low effect sizes of importance for risk assessment frameworks?, III) which of the two commonly applied model mixture reference models (CA or IA) describes metal mixture toxicity most accurately? Additionally, we proposed a framework for incorporating metal mixture toxicity in risk assessment processes.

We concluded that chronic metal mixture effects may be important, i.e. the mixture effect is on average higher than the individual effect of the most toxic metal in the mixture. This implicates that the current risks evaluation approaches based on a metal-by-metal approach (e.g. Van Sprang et al. 2009; 2016) are likely not going to be protective for communities exposed to metal mixtures. In general, the CA model was the most conservative model. Additionally, the CA model was mostly also protective at the low effect sizes that are of most importance in European risk assessment frameworks, i.e. at the EC10 level. This suggests that the CA reference model can be used as a conservative first tier in a tiered metal mixture risk evaluation scheme, although the latter requires validation for other species and preferably also at the community level. Overall, the IA model described metal mixture toxicity to *C. dubia* more accurately than the CA model.

A possible tiered metal mixture risk evaluation scheme was proposed which combined the existing bioavailability normalization-based risk assessment approaches of the individual metals (e.g. DEPA 2008; Van Sprang et al 2009; 2016) with the CA and IA mixture reference models. The proposed scheme incorporated chronic metal mixture bioavailability models to correct for the influence of water chemistry on mixture toxicity

Suggestions for further research

The development of metal mixture risk assessment frameworks is still in an initial phase. The proposed metal mixture risk assessment framework is only one of many possible options. It would be interesting to evaluate alternative approaches as well, using for instance the WHAM-

F_{Tox} bioavailability model proposed by Tipping & Lofts (2013; 2015). Clearly, the integration of metal mixture toxicity in risk assessment processes should be further investigated.

Additionally, several issues that currently hinder the application of this scheme, or possible alternatives, in metal mixture risk assessment frameworks have been identified in Chapter 8. First, to evaluate the assumptions underlying the methods used in risk assessment approaches, there is a need for studies investigating chronic metal mixture effects at the community-level, i.e. mesocosm experiment and/or field data. Second, the proposed tiered scheme integrates MMBMs in the risk assessment process. However, the lack of validated chronic MMBMs currently limits the application of these models in the scheme. Consequently, there is need for validated chronic MMBMs for different species and metal combinations. Third, metal mixture risk assessment frameworks would benefit from the uniformisation of speciation calculations. The latter would allow an evaluation of metal mixture risks at the free metal ion activity level, which is generally considered to be the most bioavailable metal fraction. In Chapter 3, we showed that recalibrating the daphnid bioavailability models of Ni, Zn, and Pb in WHAM VII resulted in similar predictive capacities as the original bioavailability models, but confirmation of this observation for the other bioavailability models currently used in the risk assessment approaches for metals is needed.

9.10 Overall contribution of the study to risk assessment

The metal (mixture) bioavailability models developed in the present study can be integrated into risk assessment frameworks, to allow an ecologically more relevant effects assessment of metals and/or metal mixtures. For instance, the chronic Pb BLM has recently been integrated in a bioavailability-based effects assessment for Pb (EC 2013b; Van Sprang et al. 2016). The chronic metal mixture toxicity data increases our overall understanding of chronic metal mixture toxicity effects. The proposed metal mixture risk evaluation scheme may guide the incorporation of metal mixture toxicity into future risk assessment frameworks.

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Summary

Metals are indispensable in our society. However, anthropogenic pollution has led worldwide to elevated metal concentrations in aquatic ecosystems. The toxicological implications of these elevated concentrations endanger natural communities worldwide. This has urged authorities all over the world to develop Environmental Quality Standards (EQS) and risk assessment approaches. However, it is well known that water chemistry characteristics, such as pH, dissolved organic carbon (DOC), and hardness, influence the bioavailability and thus the toxicity of metals. To be able to account for the effects of water chemistry on metal toxicity biotic ligand models (BLMs) or other bioavailability models have recently been developed for several metals. These models typically start from the principle that the toxicity of a metal is dependent on the concentration of the metal bound to the biotic ligand (BL), i.e., a receptor at the cell surface, and the competitive binding of certain cations (e.g., H^+, Ca^{2+}) at the biotic ligand sites. Recently, European risk assessment procedures for metals, such as Zn and Ni, have implemented bioavailability normalization based approaches and Environmental Quality Standards (EQS) for metals, such as Ni, in the European Water Framework Directive are now bioavailability based. This all has increased the ecological relevance of metal EQS-derivations and ecological risk assessment processes.

However, metals mostly occur as mixtures in the environment, but metal mixture toxicity is currently not considered by most regulatory frameworks Hence, the incorporation of metal mixture toxicity in these frameworks currently poses a new challenge for the regulatory authorities worldwide. The reproducibility of mixture toxicity by a mixture reference model is crucial for future risk assessment procedures. Two general reference models are at the moment commonly used for the description of mixture toxicity: concentration addition (CA) and independent action (IA). The CA model assumes that substances have a similar mode of action and that a substance in a mixture can be exchanged for other substances without changing the overall mixture toxicity, as long as their concentrations are *equi-effective*. The IA model assumes that the substances in a mixture have a dissimilar mode of action and the response of the mixture substances is calculated as a product of responses from each of the substances. Both reference models depart from the idea of non-interactivity, i.e. the substances in the mixture do

not interact. However, this assumptions is not always fulfilled and substances often do interact when combined in a mixture. If the observed mixture effect is larger/smaller than expected based on the reference model, the mixture acts synergistically/antagonistically.

It has been suggested that a priori knowledge of modes of action may be used to select either IA or CA in those risk assessment procedures, although this remains to be tested for chronic metal mixture toxicity. This currently hinders the development of metal mixture risk assessment frameworks. Therefore, the present study aimed to investigate chronic toxicity of Ni, Zn, and Pb mixtures to waterfleas (Daphniidae), i.e. *Ceriodaphnia dubia* and *Daphnia magna*. Different ions have been shown to compete with these metals at uptake sites, which suggests the occurrence of dissimilar modes of action between these metals. Based on the latter, we hypothesised I) that the toxicity of their binary and ternary mixtures follows the IA model rather than the CA model, and II) that toxicity of Ni-Zn-Pb mixtures under varying water-chemistry can be predicted by a simple metal mixture bioavailability model that combines the bioavailability models of each of the individual metals with the IA model. Finally, we also wanted to address the implications of our results for future metal mixture risk assessment processes.

The actual research in this study was divided into four major parts. First, we further refined and/or developed the tools (i.e. Biotic Ligand Models (BLM) or bioavailability models) used to predict individual metal toxicity. In a second part, we investigated the interactive effects of Ni, Zn, and/or Pb mixtures on chronic daphnid reproduction. In a third part of this study, we investigated whether chronic metal mixture toxicity to *C. dubia* can be predicted using a metal mixture bioavailability model. Finally, we evaluated the implications of our chronic metal mixture toxicity results for metal mixture risk assessment processes.

At the start of the present study, there were still some unresolved issues about the effects of water chemistry parameters, such as Ca and pH, on chronic Pb toxicity to *C. dubia*. In Chapter 2, we investigated therefore the individual effects of Ca and pH on chronic Pb toxicity to *C. dubia*. Ca did only influence chronic Pb toxicity to *C. dubia* to a relatively small extent, whereas a high pH (8.2) provided strong protection against Pb toxicity (compared to lower pH levels). Based on this dataset, a chronic Pb BLM for *C. dubia* was developed. The effect of pH was modeled as a single BL-site competition by H^+ with a log K_{HBL}=7.6, while no other competitive constants were needed. The developed BLM was shown in an independent validation with three

other datasets to be capable of predicting chronic Pb toxicity to different clones of *C. dubia* by an error of less than a factor of 2 in most synthetic and natural waters considered. Based on our study with *C. dubia,* BLM-based criteria are likely to be more appropriate relative to hardness-based criteria to address the risk of Pb in surface waters.

In Chapter 3, we evaluated whether the chronic daphnid single metal bioavailability models can be updated to the WHAM VII speciation software. Currently, the chronic bioavailability models for individual metals rely on different speciation software and assumptions to calculate chemical speciation in solutions. To integrate the individual metal bioavailability models in a bioavailability model for metal mixtures, an uniformisation of the speciation calculations was required. Our results show that WHAM VII (under the assumption of 65% active fulvic acid and default metal-fulvic acid binding constants) can be used as speciation model to predict metal toxicity in aquatic environments almost equally well as with the original speciation software and assumptions. However, adapting the default stability constants for inorganic complexation to those reported by the National Institute of Standards and Technology improved the overall model predictions.

In Chapter 4, we evaluated if the existing chronic *D. magna* Zn BLM can be used to predict chronic Zn toxicity to *C. dubia*. We observed that the chronic Zn *D. magna* BLM did not accurately predict Zn toxicity to *C. dubia,* even if the model was specifically calibrated on the sensitivity of *C. dubia*. Dissolved Zn toxicity to *C. dubia,* expressed as $EC50_{Zndiss}$, were predicted within a 2-fold error for only 3 out of 5 waters. Moreover, it was observed that the chronic Zn *D. magna* BLM underestimated the effect of pH on toxicity of the free Zn^{2+} ion. A preliminary *C. dubia* specific Zn bioavailability model was therefore developed. In this model the single BL-site competition effect of H^+ of the *D. magna* BLM was replaced by a log-linear pH effect. Additionally, it was assumed that the competition effects of Ca^{2+}, Mg^{2+} and Na^+ at the Zn biotic ligand site are the same for *C. dubia* and *D. magna*. The model was shown to predict chronic Zn toxicity to *C. dubia* within twofold error in natural waters in the pH range from pH 7 to 8.3. The difference in the effect of pH on Zn^{2+} toxicity between *D. magna* and *C. dubia*, two phylogenetically closely related species, deserves further attention, because of the importance of the cross-species application of bioavailability models in ecological risk assessment procedures and environmental quality standard derivations for metals.

In Chapter 5, we investigated the effects of binary Ni-Zn mixtures on *D. magna* reproduction relative to the two mixture reference models. The Ni-Zn mixture acted noninteractively according to the CA model and synergistically according to the IA model. However, the type of interactive effect was dependent on the effect size at which Ni and Zn were combined in the mixture. Weak antagonistic or non-interactive effects occurred in the mixture treatments where each of the individual metals produced insignificant or only weak adverse effects on their own (i.e. less than 20% reduction of reproductive performance). Conversely, synergistic mixture effects (according to both the CA and the IA model) only occurred when at least one of both metals in the mixture caused a greater than 20% effect on reproduction. This highlights the importance of investigating metal mixture toxicity at low effect sizes and warns against extrapolating conclusions about metal mixture interactions from high to low effect sizes. Since low effect sizes are the most relevant ones in most regulatory frameworks, our data suggest that the CA and IA mixture toxicity models can both serve as conservative models for predicting effects of Ni-Zn mixtures.

In Chapter 6, the toxicity of binary and ternary mixture combinations of Ni, Zn, and Pb on *C. dubia* reproduction was investigated. Because of anticipated differences in modes of action between these metals, we expected that the effects of their mixtures followed the IA model rather than the CA model. The toxicity of the metals in most mixtures were antagonistic relative to the CA model, whereas no significant interactive effects ($p<0.05$) were observed relative to the IA model. The only exception was the binary Pb-Zn mixture, for which mixture effects were non-interactive based on the dissolved concentrations, but antagonistic when exposure was expressed as free ion activities, all relative to the IA model. This change in interactive effects depending on the expression of exposure could be explained based on competition between Pb and Zn at the binding sites of DOC. Overall, the IA model fitted the observed toxicity better than the CA model, which was consistent with our hypothesis based on the different modes of action of these metals. The CA model mostly overestimated toxicity. We observed that the mixture effect of the Ni-Zn mixture differed between *D. magna* and *C. dubia*. The latter warns against extrapolation of the type of interactive effects between species, even when they are closely related.

In Chapter 7, we investigated whether chronic metal mixture toxicity to *C. dubia* can be predicted using a relatively simple chronic metal mixture bioavailability model (MMBM). The

MMBM was developed by combining the chronic *C. dubia* bioavailability models for Pb and Zn developed in the present study and the pre-existing Ni bioavailability model with the IA model. The metal mixture bioavailability model assumes that each metals binds to its own biotic ligand site and that Ni^{2+}, Zn^{2+} and Pb^{2+} do not compete for binding at each other's biotic ligand site. The MMBM relates metal (mixture) toxicity to the concentration of free metal ion binding to its metal-specific biotic ligand. The metal mixture bioavailability model predicted chronic toxicity of the ternary Ni-Zn-Pb mixture in 6 natural waters differing in pH, Ca, and/or dissolved organic carbon at least equally well as the chronic toxicity in the individual metal treatments. This suggests that the MMBM are a promising tool to predict chronic Ni-Zn-Pb mixture toxicity to daphnids and could eventually be used in metal risk assessment frameworks.

In conclusion, both the CA model as well as the IA model provided conservative predictions for Ni, Zn, and Pb mixture toxicity to daphnids at the low effect sizes of importance in most regulatory frameworks. However, the interactive effects of Ni, Zn, and Pb mixtures observed were variable, even for the restricted taxonomical scope (daphnids) and number of metals (Ni, Zn and Pb) considered. In Chapter 8, we combined all mixture toxicity data for *C. dubia* in a meta-analysis. Metal mixture toxicity was shown to be important, i.e. for 62% of the mixture treatments the observed mixture effect was larger than the effect of most toxic metal when applied singly. When the mixture treatments relevant for European environmental risk assessment were considered, i.e. the mixture treatments where all metals were present below their 10% effective concentration (EC10), the observed mixture effect was higher than 10% for 33% of these mixture treatments. This indicates that significant mixture effects can occur when combining metals below their EC10 levels. Hence, the current metal risk assessment approaches, which are based on a metal-by-metal evaluation, could result in underestimation of the risks on communities exposed to metal mixtures in certain situations. Of the two commonly applied mixture reference models, the CA model was generally the most conservative model. This suggests that the CA reference model can be used as a conservative first tier in a tiered metal mixture risk evaluation scheme. However, the CA model mostly overestimated metal mixture, while the IA model described metal mixture toxicity to *C. dubia* more accurately. The IA model can therefore be used as a more liberal higher tier in a sequential tiered risk assessment approach. Based on these considerations, a possible tiered metal mixture risk evaluation scheme was proposed which combined the existing bioavailability normalization-based risk assessment

approaches of the individual metals with the CA and IA models in a sequential manner. The proposed scheme incorporates chronic metal mixture bioavailability models to correct for the influence of water chemistry on mixture toxicity.

The metal (mixture) bioavailability models developed in the present study can be integrated into risk assessment frameworks. The chronic metal mixture toxicity data increases our overall understanding of chronic metal mixture effects. The proposed metal mixture risk evaluation scheme may guide the incorporation of metal mixture toxicity into future risk assessment frameworks.

Samenvatting

Metalen zijn onmisbaar in onze samenleving. Maar de productie en verwerking van metalen en de daarbij horende afvalstromen hebben wereldwijd geleid tot verhoogde concentraties metalen in aquatische ecosystemen. De toxicologische implicaties van de verhoogde metaal concentraties bedreigen de natuurlijke gemeenschappen in het aquatisch milieu. Dit heeft overheden over de hele wereld er toe aangezet om milieukwaliteitsnormen en ecologische risico-evaluatie procedures te ontwikkelen. De biobeschikbaarheid en bijgevolg ook de toxiciteit van metalen voor aquatische organismen wordt echter beïnvloed door de fysicochemische eigenschappen van de oppervlaktewaters, bv. pH, opgeloste organische koolstof (DOC) en hardheid. Om de toxiciteit van metalen onder variërende waterchemie te kunnen voorspellen, zijn er recent biotische ligand modellen (BLMs) of biobeschikbaarheidsmodellen ontwikkeld voor diverse metalen. De onderliggende assumptie van deze modellen is dat de toxiciteit van een metaal afhankelijk is van de concentratie van het metaal dat bindt ter hoogte van het biotisch ligand (BL), i.e. een receptor aan het celoppervlak, en de competitieve binding van bepaalde kationen (bijvoorbeeld H^+ en Ca^{2+}) op de BL sites. De huidige Europese risicobeoordelingsprocedures voor metalen, zoals Zn en Ni, volgen een biobeschikbaarheidsnormalisatie gebaseerde benaderingen en ook de milieukwaliteitsnormen voor metalen, zoals Ni, in de Europese Kaderrichtlijn Water laten sinds kort biobeschikbaarheidscorrecties toe. Dit alles heeft de ecologische relevantie van milieukwaliteitsnormen en ecologische risicobeoordelingsprocedures voor metalen verhoogd.

Metalen komen in het milieu echter meestal voor in mengsels; terwijl metaalmengseltoxiciteit in de huidige regelgevende procedures meestal niet in rekening wordt gebracht. De incorporatie van metaalmengseltoxiciteit in deze procedures vormt momenteel een nieuwe uitdaging voor de regelgevende instanties over de hele wereld. De voorspelbaarheid van metaalmengseltoxiciteit door een mengselreferentiemodel is van cruciaal belang voor de toekomstige risico-evaluatie procedures. De volgende twee referentiemodellen worden momenteel algemeen gebruikt voor de beschrijving van mengsel toxiciteit: concentratie additie (CA) en onafhankelijke actie (IA). Het CA model gaat er vanuit dat chemische stoffen eenzelfde werkingsmechanisme hebben en dat een stof in een mengsel kan worden uitgewisseld voor een andere equitoxische stof zonder dat de totale mengseltoxiciteit verandert. Het IA model daarentegen veronderstelt dat de stoffen in een mengsel een verschillend werkingsmechanisme hebben. De toxiciteit van een mengsel wordt voor

het IA model voorspeld op basis van de gekende effecten van de individuele componenten van het mengsel. Beide referentiemodellen gaan er van uit dat de stoffen in een mengsel niet interageren, i.e. de stoffen zijn niet-interactief. Maar deze assumptie klopt niet altijd en interacties tussen stoffen worden vaak waargenomen. Als het waargenomen mengseleffect groter/kleiner is dan verwacht op basis van het referentiemodel werkt het mengsel synergetisch/antagonistisch.

In principe kan op basis van a priori kennis van de werkingsmechanismen van de stoffen in een mengsel een selectie worden gemaakt tussen het CA of het IA model in risicobeoordelingsprocedures. De geldigheid van deze hypothese is echter tot op heden onvoldoende getest voor metaalmengsels. Dit belemmert op dit moment de integratie van metaalmengseltoxiciteit in ecologische risicobeoordelingsprocedures. Daarom was deze studie gericht op het onderzoeken van de chronische toxiciteit van Ni, Zn en Pb mengsels op watervlooien (Daphniidae), i.e. *Ceriodaphnia dubia* en *Daphnia magna*. Door de observatie dat de kationen die competitie voeren ter hoogte van de opname sites verschillen tussen Ni, Zn en Pb, veronderstelden we dat deze metalen een verschillende werkingsmechanisme hadden. Op basis daarvan veronderstelden we dat I) de toxiciteit van binaire en ternaire mengsels van Ni, Zn en Pb eerder het IA model volgt in plaats van het CA model, en II) dat toxiciteit van ternaire Ni-Zn-Pb mengsels in verschillende oppervlaktewateren kan worden voorspeld aan de hand van een relatief eenvoudig metaalmengsel biobeschikbaarheidsmodel (MMBM) dat de biobeschikbaarheidsmodellen van elk van de afzonderlijke metalen met het IA model combineert. Tot slot, wilden we ook de implicaties van onze resultaten voor toekomstige ecologische risicobeoordelingsprocessen evalueren.

Het eigenlijke onderzoek in deze studie kan worden onderverdeeld in vier delen. In een eerste deel werden de hulpmiddelen voor het voorspellen van de individuele metaal toxiciteit (i.e. BLMs of biobeschikbaarheidsmodellen) verder verfijnd en/of ontwikkeld. In een tweede deel onderzochten we de effecten van Ni, Zn en/of Pb mengsels op de reproductie van watervlooien. In een derde deel van deze studie, onderzochten we of chronische metaalmengseltoxiciteit voor de watervlo C. dubia kan voorspeld worden met behulp van een chronisch MMBM. Tenslotte evalueerden we de implicaties van chronische metaalmengseltoxiciteit voor ecologische risico-evaluatie processen.

Aan het begin van deze studie, waren de effecten van waterchemie variabelen zoals, Ca en pH, op de chronische toxiciteit van Pb voor watervlooien nog niet helemaal duidelijk. In Hoofdstuk 2, onderzochten we daarom de individuele effecten van Ca en pH op de chronische Pb toxiciteit voor *C. dubia*. Ca had slecht een gering effect op de chronische toxiciteit van Pb, terwijl een hoge pH (8,2) net de toxiciteit sterk verminderde in vergelijking met de lagere pH niveaus. Op basis van deze data werd een chronische Pb BLM voor *C. dubia* ontwikkeld. Het effect van de pH werd daarin gemodelleerd als een enkelvoudig BL-site competitie effect door H^+ (log K_{HBL}=7.6). De opname van andere competitie constanten bleek niet nodig te zijn. De predictiecapaciteit van het ontwikkelde BLM werd geëvalueerd in een onafhankelijke validatie met drie andere datasets met chronische toxiciteitsdata van verschillende *C. dubia* laboratorium populaties. Uit deze validatie bleek dat de chronische Pb toxiciteit in de meeste synthetische en natuurlijke waters werd voorspeld met minder dan een factor twee afwijking van de waargenomen toxiciteit. Op basis van onze studie met *C. dubia*, lijken BLM-gebaseerde criteria meer geschikt om de risico's van Pb in oppervlaktewaters in te schatten dan hardheid-gebaseerde criteria.

In hoofdstuk 3 onderzochten we of de individuele chronische daphnid biobeschikbaarheidsmodellen voor Ni, Zn en Pb kunnen geactualiseerd worden naar WHAM VII. Momenteel is elk chronisch biobeschikbaarheidsmodel gekoppeld aan een verschillende speciatiesoftware. Bovendien verschillen ook de assumpties om de chemische speciatie in oppervlaktewaters te berekenen tussen de modellen. Dit verhindert momenteel de integratie van deze biobeschikbaarheidsmodellen in een metaalmengselmodel. Onze resultaten toonden aan dat wanneer WHAM VII (uitgaande van 65% actief fulvozuur en de standaard metaal-fulvozuur binding constanten) als speciatiemodel word gebruikt metaaltoxiciteit in aquatische milieus vrijwel even accuraat kan voorspeld worden in vergelijking met wanneer de oorspronkelijke speciatiesoftware en assumpties worden gebruikt. Het aanpassen van de standaard stabiliteitsconstanten voor anorganische complexatie naar de waarden gerapporteerd door het 'National Institute of Standards and Technology' verbeterde echter de algemene modelvoorspellingen.

In hoofdstuk 4 onderzochten we of het bestaande chronische *D. magna* Zn BLM kan worden gebruikt om de chronische Zn toxiciteit voor *C. dubia* te voorspellen. De chronische Zn toxiciteit voor *C. dubia* werd niet accuraat voorspeld door het Zn D. magna BLM, ondanks dat het model

specifiek was gekalibreerd op de gevoeligheid van *C. dubia*. Het effect van pH op de Zn^{2+} toxiciteit bleek sterker te zijn voor *C. dubia* dan voor *D. magna*. Daarom werd een voorlopige *C. dubia*-specifiek Zn biobeschikbaarheidsmodel ontwikkeld. In dit model werd het effect van pH gemodelleerd als een log-lineair pH effect en namen we aan dat de competitie effecten van Ca^{2+}, Mg^{2+} en Na^{+} ter hoogte van het Zn biotische ligand gelijk zijn voor *C. dubia* en *D. magna*. Het *C. dubia*-specifieke model voorspelde chronische Zn toxiciteit voor *C. dubia* binnen een factor twee afwijking van de waargenomen toxiciteit in natuurlijke wateren met pH tussen pH 7 en pH 8.3.

In hoofdstuk 5 onderzochten we de effecten van binaire Ni-Zn mengsels op de reproductieve toxiciteit voor *D. magna*. De mengsel effecten van een binair Ni-Zn mengsel waren niet-interactief volgens het CA model en synergistisch volgens het IA model. Het type mengselinteractie dat werd geobserveerd was echter afhankelijk van de individuele effect groottes waarbij Ni en Zn werden gecombineerd in het mengsel. Zwakke antagonistische of niet-interactieve effecten werden geobserveerd in mengselbehandelingen waarin elk van de afzonderlijke metalen geen of slechts een klein effect veroorzaakte., i.e. indien ze beiden minder dan 20% effect hadden op de reproductie. Maar synergistische mengseleffecten (zowel volgens de CA en de IA model) traden op als ten minste een van beide metalen in het mengsel meer dan 20% effect op de voortplanting veroorzaakt. Dit benadrukt het belang van het onderzoeken van metaalmengseltoxiciteit bij lage effect concentraties en waarschuwt voor de extrapolatie van conclusies over metaalmengselinteracties van hoge naar lage effect groottes. Aangezien voor ecologische risicobeoordelingsprocessen voornamelijk de lage effect groottes van belang zijn, suggereert onze studie dat zowel het CA als het IA referentiemodel kan gebruikt worden als conservatieve modellen voor het voorspellen van de effecten van Ni-Zn mengsels.

In hoofdstuk 6 werd de toxiciteit van binaire en ternaire mengselcombinaties van Ni, Zn en Pb op de reproductie van *C. dubia* onderzocht. Op basis van de veronderstelde verschillen in werkingsmechanismen tussen deze metalen, verwachtten we dat de mengseleffecten eerder het IA model zouden volgen in plaats van het CA model. De geobserveerde mengseleffecten waren over het algemeen antagonistisch ten opzichte van het CA model, terwijl er geen significante interactieve effecten werden waargenomen ten opzichte van het IA model. De enige uitzondering was het binaire Pb-Zn mengsel, waarvoor de mengseleffecten niet-interactief waren op basis van opgeloste metaalconcentraties, maar antagonistische wanneer de blootstelling werd uitgedrukt als

vrije metaalion activiteiten, allemaal ten opzichte van de IA model. Deze verandering in geobserveerde interactieve effecten afhankelijk van de expressie van blootstelling kan worden verklaard op basis van competitie tussen Pb en Zn voor de bindingsplaatsen van DOC. Het IA model voorspelde de waargenomen toxiciteit over het algemeen beter dan het CA model, wat in overeenstemming is met onze hypothese gebaseerd op de verschillende werkingsmechanismen van deze metalen. Het CA model overschatte doorgaans de toxiciteit. De geobserveerde effecten van het binaire Ni-Zn mengsel verschilden tussen *D. magna* en *C. dubia*. Dit laatste wijst er op dat mengseleffecten tussen soorten niet altijd geëxtrapoleerd kunnen worden, zelfs wanneer de soorten nauw verwant zijn.

In hoofdstuk 7 onderzochten we of chronische metaalmengseltoxiciteit voor C. dubia kan voorspeld worden aan de hand van een relatief eenvoudig metaalmengsel biobeschikbaarheidsmodel (MMBM). We ontwikkelden een MMBM voor Ni, Zn en Pb mengsels door de chronische *C. dubia* biobeschikbaarheidsmodellen voor Pb en Zn ontwikkeld in het huidige onderzoek en het bestaande Ni biobeschikbaarheidsmodel te combineren met het IA mengselreferentie model. Het MMBM gaat ervan uit dat elk metaal bindt aan zijn eigen biotische ligand en dat Ni^{2+}, Zn^{2+} en Pb^{2+} geen competitie voeren voor binding ter hoogte van elkaars biotisch ligand. In het model wordt metaal(mengsel)toxiciteit gerelateerd aan de concentratie van vrije metaalionen die binden aan het metaal-specifieke biotisch ligand. Het MMBM voorspelde de chronische toxiciteit van het ternaire Ni-Zn-Pb mengsel in 6 natuurlijke wateren, die verschillen in pH, Ca en/of opgeloste organische koolstof concentratie, ten minste even nauwkeurig als de chronische toxiciteit van de individuele metalen. Dit suggereert dat het MMBM een veelbelovend instrument is om de chronische toxiciteit van Ni-Zn-Pb mengsel voor watervlooien te voorspellen en dat deze modellen uiteindelijk ook potentieel kunnen worden gebruikt in risico-evaluatie processen voor metaalmengsels.

Zowel het CA model, als het IA model gaven conservatieve voorspellingen van Ni, Zn en Pb mengseltoxiciteit voor de lage effect niveaus die het meest van belang zijn in ecologische risicobeoordelingsprocessen. De waargenomen interactieve effecten in Ni, Zn, Pb en mengsels waren echter variabel, zelfs voor de beperkte taxonomische reikwijdte (watervlooien) en het beperkt aantal metalen (Ni, Zn en Pb) beschouwd in deze studie. In hoofdstuk 8, combineerden we daarom alle mengseltoxiciteitsgegevens voor *C. dubia* in een meta-analyse. Metaalmengseltoxiciteit bleek belangrijk te zijn, aangezien voor 62% van de

mengselbehandelingen het waargenomen mengsel effect groter was dan het individuele effect van het meeste toxische metaal in het mengsel. In de Europese ecologische risicobeoordelingsprocessen zijn voornamelijk de mengsels waar het individuele effect van het meest toxische metaal lager is dan 10% van belang. Wanneer deze mengselbehandelingen werden beschouwd was voor 33% het waargenomen mengseleffect hoger dan 10%. Dit geeft aan dat significante mengseleffecten ook kunnen optreden wanneer metalen worden gecombineerd onder hun individuele EC10 niveau. Daardoor zouden de huidige risicobeoordelingsbenaderingen voor metalen in bepaalde situaties kunnen leiden tot een onderschatting van de risico's voor ecologische gemeenschappen blootgesteld aan metaalmengsels. Het CA model was over het algemeen het meest conservatieve model van de twee mengselreferentiemodellen. Dit suggereert dat het CA referentiemodel kan worden gebruikt als een conservatieve eerste tier in een sequentiële metaalmengsel risico-evaluatie proces. Het CA model overschat echter meestal de metaalmengseltoxiciteit, terwijl het IA model metaalmengseltoxiciteit voor *C. dubia* nauwkeuriger voorspelde. Het IA model kan bijgevolg gebruikt worden in hogere tiers van het sequentiële metaalmengsel risico-evaluatie proces. Op basis van deze overwegingen werd een mogelijke sequentieel metaalmengsel risicobeoordelingsschema voorgesteld waarin de bestaande biobeschikbaarheidsgebaseerde risico-evaluatieprocessenvoor de individuele metalen op een sequentiële wijze gecombineerd werden met de CA en IA-modellen. Het voorgestelde schema bevat chronische metaalmengsel biobeschikbaarheidsmodellen die rekening houden met het effect van waterchemie op de mengseltoxiciteit.

De biobeschikbaarheidsmodellen voor metalen ontwikkeld in deze studie kunnen worden geïntegreerd in de risicobeoordelingsprocessen voor metalen. De chronische metaalmengsel toxiciteitsdata verhoogt de algemene kennis van chronische metaalmengseltoxiciteit. Het voorgestelde metaalmengsel risico-evaluatie schema kan helpen bij het opstellen van toekomstige risico-evaluatie processen voor metaalmengsels.

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Curriculum Vitae

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Nys C, Janssen CR, De Schamphelaere KAC. The effects of pH on acute and chronic toxicity of Pb^{2+} on *Daphnia magna* and *Ceriodaphnia dubia* and Biotic Ligand Model (BLM) development. 22nd SETAC Europe Conference, 6th SETAC World Congress, 15-19 May, 2012 Berlin, Germany.

Developed bioavailability normalisation tools

Implementation of bioavailability models in chronic Pb bioavailability normalization tool: Arche, Ghent University, KTH and ILRZO. 2015. The Lead BLM SSD Normalization Tool. Version 1.0.0. March 2015. Available on: http://www.leadBLM.com.

Development of chronic Ni bioavailability normalization tool for pH up to 8.7. Enclosed as Supplemental Data in Nys et al. (accepted) (DOI:10.1002/etc.3232).

Educational activities

Tutoring master thesis students

Niels Verdoodt. 2014-2015. Thesis: Experimentele validatie van biotisch ligand model voorspellingen voor mengseltoxiciteit van metalen. Master of Science in Bioscience Engineering, Faculty of Bioscience Engineering, Ghent University. Promotor: prof. dr. ir. Karel De Schamphelaere; prof. dr. Colin Janssen. Tutor: Tina Van Regenmortel and Charlotte Nys

Robin Van Den Fonteyne. 2014. Thesis: Mengseltoxiciteit van Pb, Cd, en Zn mengsels bij *Ceriodaphnia dubia.* Master of Environmental Sanitation, Faculty of Bioscience Engineering, Ghent University. Promotor: prof. dr. ir. Karel De Schamphelaere; prof. dr. Colin Janssen. Tutor: Charlotte Nys

Iris Castel. 2013. Thesis: Biobeschikbaarheid van Cd, Pb en Cd-Pb mengsels voor *Ceriodaphnia dubia* in oppervlaktewater. Master of Environmental Sanitation, Faculty of Bioscience Engineering, Ghent University. Promotor: prof. dr. ir. Karel De Schamphelaere; prof. dr. Colin Janssen. Tutor: Charlotte Nys

Emely Vandewaetere. 2012-2013 Thesis: Ontwikkeling van een Biotisch Ligand Model (BLM) voor mengseltoxiciteit van metalen in oppervlaktewater. Master of Science in Bioscience Engineering, Faculty of Bioscience Engineering, Ghent University. Promotor: prof. dr. ir. Karel De Schamphelaere; prof. dr. Colin Janssen. Tutor: Charlotte Nys

Teaching activities

Guidance of practical courses and technical lectures for the course Ecological Risk Assessment.

Appendix A: Chapter 2: Development and validation of a biotic ligand model for predicting chronic toxicity of lead (Pb) to *Ceriodaphnia dubia*

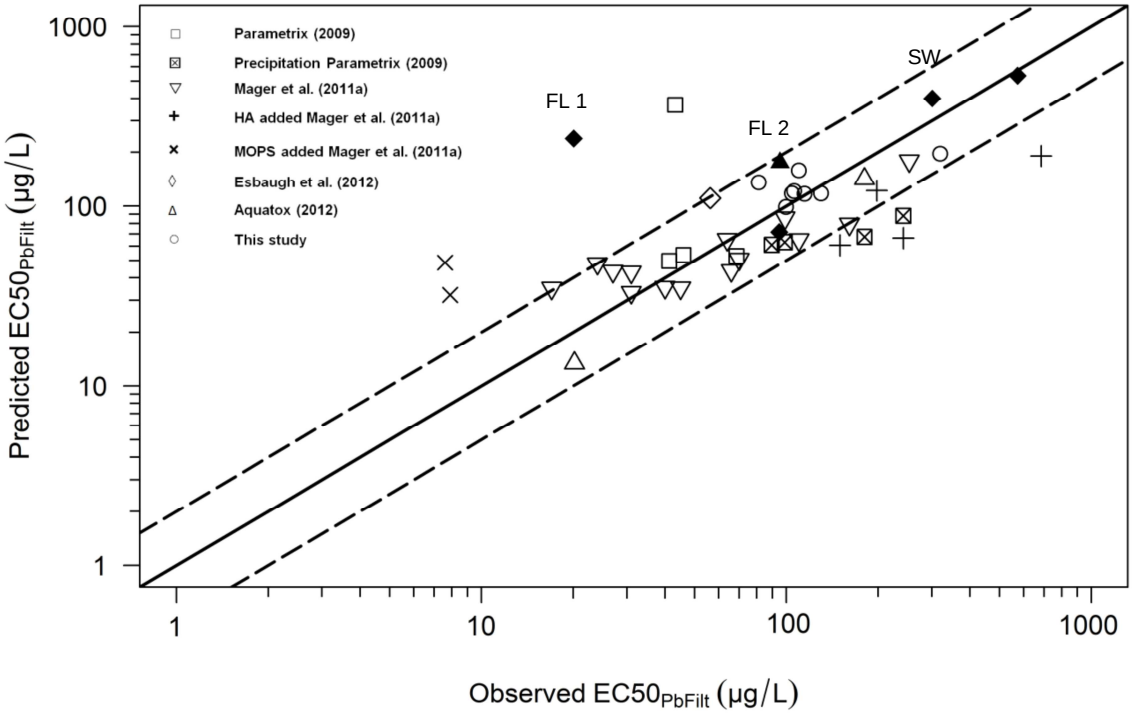

Figure A.1. Predicted versus observed reproductive toxicity of Pb to *Ceriodaphnia dubia,* expressed as EC50 (µg filtered Pb/L) for the preliminary BLM for *C. dubia* from Esbaugh et al. (2012) calibrated with the clone-specific intrinsic sensitivity. Predictions were made using Equation 1 from Esbaugh et al. (2012) linked to Visual Minteq. The dashed line represents a difference of a factor of two between the observed and predicted data. The full line represents a perfect fit between observed and predicted data. Open datapoints are from synthetic mediums, filled from natural waters. Crossed symbols represent data points where precipitation is predicted by speciation calculations. The symbols are designated as follows: □ Parametrix (2010), ⊠ waters where precipitation was predicted (Parametrix 2010), ▽ Mager et al. (2011a), + HA added media (Mager et al. 2011a), x MOPS added media (Mager et al. 2011a), ◊ Esbaugh et al. (2012), Δ AquaTox (2012), ○ data from this study. Individual test results discussed in the text are: FL1 – French Lake 1 (Esbaugh et al. 2012); FL2 – French Lake 2 (AquaTox 2012), SW – Sweetwater Strand (Esbaugh et al. 2012).

Table A.1 Visual minteq input of chronic Pb reproductive toxicity data for *Ceriodaphnia dubia* used for the BLM development (data from the present study) and for the independent validation

Source	ID	Temp ℃	pH	DOC mg/L	HA g/L	FA g/L	Na mol/L	Mg mol/L	K mol/L	Ca mol/L	Cl mol/L	SO4 mol/L	CO3 mol/L	MOPS mol/L	$EC50_{Pbdiss}$ µg/L	$EC20_{Pbdiss}$ µg/L
Parametrix 2010	Ca025	25	7.55	0.70	1.00E-06	9.10E-04	4.00E-04	1.17E-04	1.94E-04	3.27E-04	1.94E-04	3.42E-04	4.25E-04	0.00E+00	46	21
	Ca100	25	8.15	0.50	1.00E-06	6.50E-04	1.50E-03	3.40E-04	1.64E-04	9.93E-04	1.73E-04	1.08E-03	1.74E-03	0.00E+00	99	34
	Ca175	25	8.3	0.50	1.00E-06	6.50E-04	3.28E-03	6.21E-04	1.82E-04	1.69E-03	1.56E-04	2.11E-03	3.29E-03	0.00E+00	242	90
	Ca225	25	8.1	0.50	1.00E-06	6.50E-04	2.26E-03	7.16E-04	1.57E-04	2.01E-03	2.13E-04	2.24E-03	2.43E-03	0.00E+00	181	65
	pH60	25	6.05	0.50	1.00E-06	6.50E-04	6.26E-04	1.25E-04	7.98E-05	3.79E-04	4.91E-04	4.41E-04	1.20E-03	0.00E+00	69	42
	pH70	25	7	0.50	1.00E-06	6.50E-04	5.61E-04	1.24E-04	8.21E-05	3.72E-04	1.88E-04	4.47E-04	5.88E-04	0.00E+00	43	27
	pH80	25	8	0.51	1.00E-06	6.63E-04	5.74E-04	1.23E-04	7.88E-05	3.67E-04	8.83E-05	3.85E-04	5.70E-04	0.00E+00	41	26
	pH86	25	8.5	0.50	1.00E-06	6.50E-04	6.44E-04	1.34E-04	8.16E-05	4.19E-04	2.21E-04	4.19E-04	7.15E-04	0.00E+00	90	68
Mager et al. 2011	base B	25	7.2	2.18	1.00E-06	2.84E-03	7.21E-04	1.10E-04	5.40E-05	2.36E-04	9.54E-04	7.30E-05	1.22E-04	0.00E+00	64	45
	ca05	25	7.6	1.20	1.00E-06	1.56E-03	3.78E-04	4.90E-05	2.70E-05	4.63E-04	4.64E-04	3.51E-04	4.00E-04	0.00E+00	66	46
	ca16	25	7.3	1.20	1.00E-06	1.56E-03	3.85E-04	5.50E-05	3.00E-05	1.62E-03	4.59E-04	1.15E-03	4.00E-04	0.00E+00	31	17
	ca35	25	7.5	1.20	1.00E-06	1.56E-03	5.23E-04	6.80E-05	3.70E-05	3.47E-03	7.42E-04	2.79E-03	4.00E-04	0.00E+00	17	12
	ca50	25	7.5	1.20	1.00E-06	1.56E-03	5.59E-04	6.00E-05	3.40E-05	5.05E-03	5.29E-04	4.88E-03	4.00E-04	0.00E+00	40	22
	nom2	25	7.5	2.52	1.00E-06	3.28E-03	4.72E-04	5.70E-05	2.70E-05	2.39E-04	4.11E-04	2.20E-05	4.00E-04	0.00E+00	99	71
	nom4	25	7.4	5.40	1.00E-06	7.02E-03	4.96E-04	6.00E-05	3.70E-05	2.34E-04	5.07E-04	6.10E-05	4.00E-04	0.00E+00	253	176
	ha2	25	7.4	2.04	1.68E-03	1.56E-03	3.47E-04	4.30E-05	2.50E-05	1.76E-04	3.70E-04	4.80E-05	4.00E-04	0.00E+00	150	96
	ha4	25	7.3	2.52	2.64E-03	1.56E-03	3.97E-04	5.10E-05	2.60E-05	2.07E-04	3.72E-04	3.80E-05	4.00E-04	0.00E+00	242	85
	ha8	25	7.4	4.92	7.44E-03	1.56E-03	4.69E-04	4.90E-05	2.60E-05	1.88E-04	4.21E-04	4.70E-05	4.00E-04	0.00E+00	198	53
	ha32	25	7.3	7.16	9.96E-03	2.84E-03	8.81E-04	1.14E-04	5.60E-04	2.51E-04	1.07E-03	8.30E-04	2.35E-04	0.00E+00	685	523
	pH6.4(MOPS)	25	6.4	1.20	1.00E-06	1.56E-03	1.08E-03	4.90E-05	2.90E-05	1.71E-04	4.33E-04	4.40E-05	4.00E-04	4.00E-03	8	4
	pH7.2(MOPS)	25	7.3	1.20	1.00E-06	1.56E-03	2.83E-03	5.50E-05	3.10E-05	1.76E-04	4.33E-04	4.40E-05	4.00E-04	4.00E-03	8	5
	MgSO4	25	7.4	1.20	1.00E-06	1.56E-03	3.61E-04	1.16E-03	2.40E-05	1.85E-04	3.66E-04	1.01E-03	4.00E-04	0.00E+00	45	26
	k2SO4	25	7.1	1.20	1.00E-06	1.56E-03	4.11E-04	5.20E-05	1.18E-03	2.06E-04	4.17E-04	5.66E-04	4.00E-04	0.00E+00	31	21
	NaCl15	25	7.2	1.20	1.00E-06	1.56E-03	1.65E-03	4.70E-05	2.80E-05	1.85E-04	1.43E-03	4.50E-05	4.00E-04	0.00E+00	27	19
	NaHCO3_07	25	7.7	1.20	1.00E-06	1.56E-03	7.11E-04	5.10E-05	2.50E-05	1.97E-04	4.44E-04	5.10E-05	7.00E-04	0.00E+00	70	40
	NaHCO3_19	25	7.9	1.20	1.00E-06	1.56E-03	1.78E-03	5.00E-05	2.80E-05	2.00E-04	4.68E-04	5.20E-05	1.90E-03	0.00E+00	110	69
	NaHCO3_25	25	8.2	1.20	1.00E-06	1.56E-03	2.44E-03	5.10E-05	3.00E-05	2.06E-04	5.74E-04	5.70E-05	2.50E-03	0.00E+00	161	73
	Na2SO4	25	7.3	1.20	1.00E-06	1.56E-03	1.62E-03	4.70E-05	2.70E-05	1.81E-04	3.74E-04	5.57E-04	4.00E-04	0.00E+00	24	16
Esbaugh et al. 2012	Reference	24.6	7.91	2.66	1.00E-06	3.46E-03	5.58E-04	7.30E-05	6.50E-05	2.52E-04	6.43E-04	5.90E-05	3.72E-04	0.00E+00	56	35
	Green Cove	26.1	8.05	1.84	1.00E-06	2.39E-03	2.50E-04	7.35E-04	7.30E-05	8.37E-04	1.95E-04	5.33E-04	1.76E-03	0.00E+00	95	23
	Sweetwater strand	24.2	8.47	9.61	1.00E-06	1.25E-02	4.27E-04	9.30E-05	5.50E-05	2.14E-03	3.68E-04	1.60E-05	3.77E-03	0.00E+00	301	97
	South Carolina Lake	26.4	7.31	17.32	1.00E-06	2.25E-02	2.46E-04	5.90E-05	8.90E-05	2.16E-04	2.29E-04	2.70E-05	2.23E-04	0.00E+00	573	223
	French Lake	26.2	7.07	8.23	1.00E-06	1.07E-02	1.57E-04	4.90E-05	4.90E-05	1.14E-04	1.28E-04	2.20E-05	1.17E-04	0.00E+00	20	12
Aquatox 2012	french Lake 2	25	7.1	6.80	1.00E-06	8.84E-03	1.17E-04	4.53E-05	5.12E-05	8.98E-05	1.41E-04	5.21E-06	2.12E-04	0.00E+00	95	53
	French lake reconstituted	25	6.8	5.90	1.00E-06	7.67E-03	2.17E-04	4.53E-05	5.12E-05	8.73E-05	1.97E-04	8.33E-05	2.44E-04	0.00E+00	180	23
	Lab water	25	7.5	0.40	1.00E-06	5.20E-04	2.74E-04	3.74E-04	2.56E-05	4.49E-04	5.64E-04	1.25E-04	1.24E-03	0.00E+00	20	9
This study	Ca 0.25 mM	25	7.04	3.97	1.00E-06	5.17E-03	3.17E-04	1.58E-04	0.00E+00	2.44E-04	4.14E-04	1.02E-04	2.02E-04	0.00E+00	81	64
	Ca 1 mM	25	7.01	3.86	1.00E-06	5.02E-03	3.17E-04	1.58E-04	0.00E+00	8.88E-04	1.91E-03	1.02E-04	1.72E-04	0.00E+00	104	0
	Ca 1.75 mM	25	7.04	3.85	1.00E-06	5.00E-03	3.08E-04	1.58E-04	0.00E+00	1.26E-03	3.41E-03	1.02E-04	1.75E-04	0.00E+00	130	112
	Ca 2.5 mM	25	7.07	3.86	1.00E-06	5.02E-03	3.04E-04	1.58E-04	0.00E+00	1.80E-03	4.91E-03	1.02E-04	1.94E-04	0.00E+00	115	0
	pH 6.4	25	6.35	3.30	1.00E-06	4.29E-03	2.89E-03	1.58E-04	0.00E+00	7.45E-04	2.03E-03	1.40E-03	7.09E-05	0.00E+00	100	79
	pH 7	25	6.94	3.33	1.00E-06	4.33E-03	2.90E-03	1.58E-04	0.00E+00	7.69E-04	1.93E-03	1.40E-03	1.73E-04	0.00E+00	106	81
	pH 7.6	25	7.55	3.32	1.00E-06	4.32E-03	2.96E-03	1.58E-04	0.00E+00	7.35E-04	1.91E-03	1.20E-03	5.32E-04	0.00E+00	110	80
	pH 8.2	25	8.14	3.20	1.00E-06	4.17E-03	2.93E-03	1.58E-04	0.00E+00	7.43E-04	1.95E-03	1.02E-04	2.33E-03	0.00E+00	320	153

Table A.2: Mean clone-specific and mean overall intrisic sensitivities for *Ceriodaphnia dubia* for the EC20 and EC50.

BLM type	Intrinsic sensitivity type	Clone dependent intrinsic sensitivity				Overall intrinsic sensitivity
		Ugent-clone	Umiami-clone	Parametrix-clone	AquaTox-clone	
H^+ competition BLM ('Final BLM')	$EC20^*_{Pb2+}$ (nmol/L)	1.01	0.44	1.40	0.17	0.54
	$EC50^*_{Pb2+}$ (nmol/L)	1.73	1.06	3.06	0.95	1.33
H^+ and Ca^{2+} competition BLM	$EC50^*_{Pb2+}$ (nmol/L)	1.40	0.82	2.74	0.87	1.08
Preliminary BLM (Esbaugh et al (2012)	$Q50_{Pb2+}$	-2.90	-3.09	-2.61	-3.21	-3.00

Table A.3: Prediction errors on chronic Pb toxicity to *Ceriodaphnia dubia* predicted with the BLM calibrated with the overall intrinsic sensitivity.

	EC50				EC20			
	All synthetic waters	Synthetic waters without HA & MOPS additions	Field waters	Field waters without FL1	All synthetic waters	Synthetic waters without HA & MOPS additions	Field waters	Field waters without FL1
Mean prediction error	2.11	1.72	3.37	1.57	2.14	1.79	4.37	2.65
Minimum prediction error	1.00	1.00	1.03	1.03	1.01	1.01	1.38	1.38
Maximum prediction error	6.61	4.03	12.26	2.54	5.44	4.34	11.25	4.53
% Predicted within 2-fold error	58	68	40	50	61	72	20	25

Table A.4: Prediction errors on chronic Pb toxicity to *Ceriodaphnia dubia* predicted with the BLM calibrated with the clone-specific intrinsic sensitivities.

	EC50				EC20			
	All synthetic waters	Synthetic waters without HA & MOPS additions	Field waters	Field waters without FL1	All synthetic waters	Synthetic waters without HA & MOPS additions	Field waters	Field waters without FL1
Mean prediction error	1.89	1.44	3.37	1.57	1.89	1.48	3.65	2.10
Minimum prediction error	1.05	1.05	1.09	1.09	1.01	1.01	1.05	1.05
Maximum prediction error	5.70	2.16	10.58	2.22	5.45	2.27	9.86	3.99
% Predicted within 2-fold error	77	92	60	75	74	88	40	50

Table A.5: Prediction errors on chronic Pb toxicity to *Ceriodaphnia dubia* predicted with the preliminary BLM of Esbaugh et al. (2010), the final BLM developed in this study, and the H^+ and Ca^{2+} competition BLM, all calibrated with the clone-specific intrinsic sensitivities[a].

	Preliminary BLM (Esbaugh et al. 2012)	Final BLM (present study)	H^+ and Ca^{2+} competition BLM
	EC50	EC50	EC50
Mean prediction error	1.53	1.40	1.42
Minimum prediction error	1.01	1.04	1.00
Maximum prediction error	2.74	2.22	3.31
% Predicted within 2-fold error	86	92	92

[a] all data except the media where MOPS or HA was added and the French Lake 1 natural water

Table A.6 Observed & predicted ECxs for *Cerdiodaphnia dubia* and prediction errors for all waters used for BLM development and validation. Predictions were made with the developed BLM (calibrated with the overall and clone-specific intrinsic sensitivities) and the preliminary Pb BLM of Esbaugh et al. (2012) calibrated with the clone-specific sensitivity

					***C. dubia* BLM developed in the present study**								**Preliminary *C. dubia* BLM**	
					EC20				**EC50**				**EC50**	
					Overall intrinsic sensitivity		**Clone-specific sensitivity**		**Overall intrinsic sensitivity**		**Clone-specific sensitivity**		**Clone-specific sensitivity**	
Source	**ID**	**Observed EC10 (µg/L)**	**Observed EC20 (µg/L)**	**Observed EC50 (µg/L)**	**Predicted EC20 (µg/L)**	**Prediction error**	**Predicted EC20 (µg/L)**	**Prediction error**	**Predicted EC50 (µg/L)**	**Prediction error**	**Predicted EC50 (µg/L)**	**Prediction error**	**Predicted EC50 (µg/L)**	**Prediction error**
Parametrix 2010	Ca025		21	46	15	1.40	29	1.37	28	1.63	49	1.06	54	1.16
	Ca100		34	99	23	1.49	46	1.35	45	2.21	82	1.21	63	1.56
	Ca175		90	242	34	2.61	73	1.23	70	3.45	136	1.78	88	2.74
	Ca225		65	181	22	2.89	47	1.39	45	4.03	85	2.12	67	2.67
	pH60		42	69	14	3.06	31	1.34	30	2.32	62	1.11	53	1.30
	pH70		27	43	9	2.99	18	1.49	17	2.51	31	1.38	37	1.17
	pH80		26	41	18	1.43	34	1.33	33	1.27	56	1.36	50	1.20
	pH86		68	90	33	2.02	62	1.08	60	1.49	102	1.14	61	1.47
Mager et al. 2011	base B		45	64	37	1.22	32	1.39	67	1.05	58	1.11	65	1.02
	ca05		46	66	26	1.77	23	2.02	47	1.40	41	1.62	43	1.54
	ca16		17	31	19	1.09	16	1.05	34	1.11	29	1.05	33	1.07
	ca35		12	17	20	1.69	18	1.48	38	2.21	32	1.89	35	2.06
	ca50		22	40	21	1.07	18	1.23	38	1.05	33	1.23	35	1.13
	nom2		71	99	51	1.39	45	1.59	92	1.08	79	1.25	86	1.15
	nom4		176	253	104	1.69	91	1.93	187	1.35	162	1.57	179	1.42
	ha2		96	150	35	2.74	31	3.12	64	2.36	55	2.74	61	2.47
	ha4		85	242	38	2.24	33	2.56	69	3.52	59	4.09	67	3.64
	ha8		53	198	71	1.33	62	1.17	127	1.55	110	1.80	122	1.63
	ha32		523	685	109	4.79	96	5.45	197	3.48	170	4.04	190	3.60
	pH6.4(MOPS)		4	8	20	5.23	18	4.54	39	4.91	33	4.18	32	4.07
	pH7.2(MOPS)		5	8	28	5.44	24	4.77	50	6.61	43	5.70	49	6.40
	MgSO4		26	45	20	1.30	17	1.49	37	1.22	32	1.42	35	1.28
	k2SO4		21	31	24	1.15	21	1.01	44	1.43	38	1.23	43	1.39
	NaCl15		19	27	24	1.29	21	1.13	45	1.65	38	1.42	43	1.61
	NaHCO3_07		40	70	32	1.24	28	1.42	58	1.20	50	1.39	51	1.38
	NaHCO3_19		69	110	46	1.51	40	1.73	83	1.33	71	1.54	65	1.69
	NaHCO3_25		73	161	69	1.06	61	1.21	124	1.30	107	1.50	79	2.03
	Na2SO4		16	24	27	1.71	24	1.50	50	2.07	43	1.78	48	2.00
Esbaugh et al. 2012	Reference		35	56	79	2.23	69	1.96	140	2.49	121	2.16	111	1.97
	Green Cove		23	95	55	2.45	48	2.14	101	1.07	87	1.09	72	1.32
	Sweetwater strand		97	301	438	4.53	386	3.99	766	2.54	668	2.22	400	1.33
	South Carolina Lake		223	573	308	1.38	270	1.21	554	1.03	479	1.20	536	1.07
	French Lake		12	20	136	11.25	119	9.86	246	12.26	212	10.58	239	11.90
Aquatox 2012	french Lake 2		53	95	119	2.23	56	1.05	214	2.25	170	1.79	175	1.84
	French lake reconstituted		23	180	100	4.34	47	2.03	181	1.00	144	1.26	142	1.27
	Lab water		9	20	9	1.01	4	2.27	18	1.14	14	1.49	13	1.51
This study	Ca 0.25 mM	55	64	81	58	1.11	87	1.36	105	1.29	124	1.53	135	1.67
	Ca 1.00 mM	-	-	104	50	-	76	-	91	1.15	108	1.04	118	1.13
	Ca 1.75 mM	102	112	130	49	2.26	75	1.49	91	1.44	108	1.21	117	1.11
	Ca 2.50 mM	-	-	115	49	-	75	-	90	1.28	107	1.07	117	1.02
	pH 6.4	70	79	100	47	1.66	73	1.08	89	1.12	106	1.07	99	1.01
	pH 7.0	69	81	106	52	1.57	79	1.03	94	1.12	112	1.06	121	1.14
	pH 7.6	67	80	110	72	1.11	109	1.36	130	1.18	153	1.39	158	1.44
	pH 8.2	77	153	320	123	1.24	184	1.20	219	1.46	258	1.24	196	1.63

Table A.7 Observed & Predicted $EC10_{Pbfilt}$ (µg filtered Pb/L) in the Brisy water for *Ceriodaphnia dubia*. Predictions were made with the developed BLM (calibrated with the clone-specific intrinsic sensitivities)

	Observed EC10 (µg/L)	Predicted EC10 (µg/L)	Prediction error
Ca 0.25 mM	55	71	1.30
Ca 1.00 mM	-	62	-
Ca 1.75 mM	102	62	1.66
Ca 2.50 mM	-	61	-
pH 6.4	70	60	1.18
pH 7.0	69	64	1.07
pH 7.6	67	89	1.34
pH 8.2	77	152	1.54

Appendix B: Chapter 5: Mixture toxicity of nickel and zinc to *Daphnia magna* is non-interactive at low effect sizes, but becomes synergistic at high effect sizes

Table B.1. Physico-chemical composition of the test medium used in the AHA- and natural DOC test series to investigate binary Ni-Zn mixture (21d-)reproductive toxicity to *Daphnia magna*.

Medium	Temp (°C)	pH	Colloidal Fulvic Acid (mg/L)	Colloidal Humic Acid (mg/L)	Na (mM)	Mg (mM)	K (mM)	Ca (mM)	Mn (µM)	Fe (µM)	Cu (µM)	Cl (mM)	SO_4 (mM)	DIC (mM)
AHA	20	7.8	-	1.60	0.90	0.74	0.08	1.91	2.86	6.55	0.12	3.91	0.74	0.77
Natural DOC	20	7.8	7.00		0.86	0.35	0.08	1.37	1.34	1.87	0.10	3.91	0.74	0.69

DIC= Dissolved organic carbon

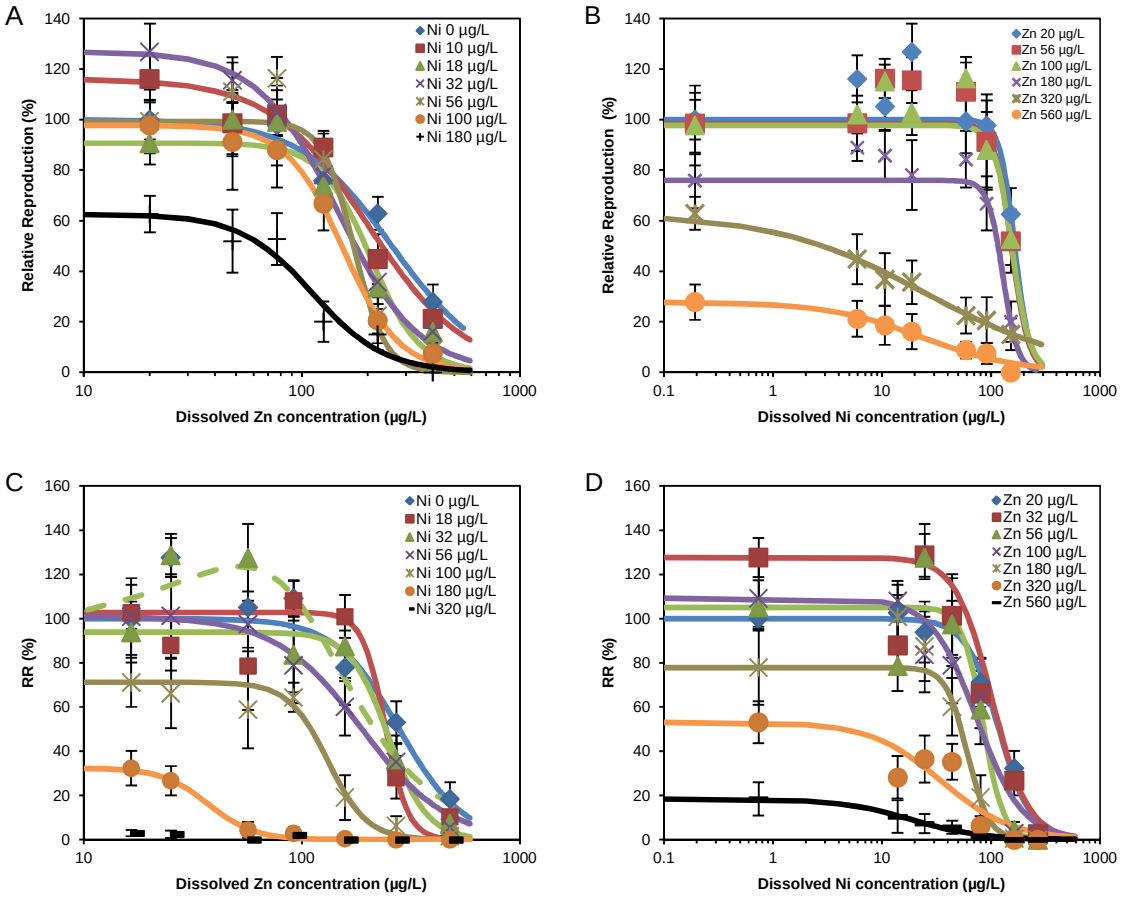

Figure B.1. Concentration response data for relative reproduction for *Daphnia magna*(%; symbols) and fitted log-logistic concentration response curves (Eq. 5.3 of main paper; full lines) under different Ni treatments in function of dissolved Zn concentrations for the AHA- (A) and natural DOC-test series (C) or under different Zn treatments in function of dissolved Ni concentrations for the AHA- (B) and natural DOC-test series (D). Error bars indicate standard errors (n=10). Concentration response curve parameters are reported in Table B.2. The concentration response data was checked for hormesis effects with the method of Van Ewijk & Hoekstra (1993). Concentration response curves with significant hormesis are indicated with a dashed line.

Table B.2A. Overview of parameters of the concentration response curves of Ni at different Zn concentrations for 21d-reproductive toxicity to *Daphnia magna*. EC50 and EC10, expressed as dissolved Ni concentrations and Ni^{2+} activities, and the slopes of the concentration response curves are reported. The individual relative reproduction of every Zn treatment at Ni 0 µg/L is also reported (values higher than 100% indicate a stimulation in reproduction relative to the control). 95% confidence intervals on effect concentrations are reported between brackets.

	Nominal Zn (µg/L)	Total Zn of fresh medium (µg/L)	Dissolved Zn of fresh medium (µg/L)	Dissolved Zn of old medium (µg/L)	Mean dissolved Zn of fresh and old medium (µg/L)	Wham VI estimated Zn^{2+} activity (nmol/L)	Relative reproduction at Ni 0 µg/L (%)	$EC10_{Nidiss}$ (µg/L)	$EC50_{Nidiss}$ (µg/L)	β_{Nidiss}	$EC10_{Ni2+}$ (nmol/L)	$EC50_{Ni2+}$ (nmol/L)	β_{Ni2+}
Natural DOC	**Zn 20**	18±3	18±4	15±~3	17±4	51	100	55 (48-64)	118 (110-127)	2.90	436 (371-512)	1035 (945-1114)	2.70
	Zn 32	31±5	29±5	21±5	25±7	81	128	28 (16-48)	82 (63-107)	2.04	215 (119-389)	690 (518-919)	1.88
	Zn 56	70±6	67±5	46±5	57±12	210	105	53 (27-103)	84 (66-107)	4.70	415 (204-842)	687 (523-903)	4.35
	Zn 100	107±5	105±4	79±4	92±14	376	109	26 (15-45)	74 (57-96)	2.07	199 (108-365)	619 (469-819)	1.93
	Zn 180	177±8	172±5	143±9	157±29	737	78	37[ac] (20-67)	61[b] (45-82)	4.42	299 (173-518)	495 (378-647)	4.37
	Zn 320	298±6	291±5	252±13	271±30	1441	53	7 (2-25)	35 (22-57)	1.34	40 (9-181)	264 (151-460)	1.17
	Zn 560	507±9	502±7	453±22	478±29	2871	18	4 (0.2-70)	18 (7-49)	1.34	19 (0.6-598)	127 (40-400)	1.17
Aldrich Humic Acid	**Zn 20**	25±4	25±5	15±5	21±8	129	100	116 (54-249)	165 (124-219)	6.29	1182 (548-2548)	1652 (1262-2242)	6.25
	Zn 56	64±8	61±8	35±14	48±19	329	98	104 (53-207)	155 (128-187)	5.57	1059 (529-2117)	1577 (1303-1909)	5.51
	Zn 100	102±12	97±13	57±13	77±27	551	98	102 (53-197)	156 (128-191)	5.19	1039 (536-2012)	1592 (1299-1952)	5.15
	Zn 180	165±9	154±12	98±17	126±37	936	76	91[a] (57-144)	129[a] (104-159)	6.28	919 (576-1467)	1309 (1057-1621)	6.22
	Zn 320	281±13	267±16	179±36	223±62	1740	63	0.7 (0.1-8.8)	25 (12-53)	0.63	7.4[b] (0.6-88)	249 (116-536)	0.62
	Zn 560	487±16	466±23	332±55	399±99	3244	28	2.5 (0.3-21.9)	23 (10-55)	0.99	25 (3-219)	233 (98-556)	0.99

Table B.2.B. Overview of parameters of the concentration response curves of Zn at different Ni concentrations for 21d-reproductive toxicity to *Daphnia magna*. EC50 and EC10, expressed as dissolved Zn concentrations and Zn^{2+} activities, and the slopes of the concentration response curves are reported. The individual relative reproduction of every Ni treatment at Zn 20 µg/L is also reported (values higher than 100% indicate a stimulation in reproduction relative to the control). 95% confidence intervals on effect concentrations are reported between brackets.

	Nominal Ni (µg/L)	Total Ni of fresh medium (µg/L)	Dissolved Ni of fresh medium (µg/L)	Dissolved Ni of old medium (µg/L)	Mean dissolved Zn of fresh and old medium (µg/L)	Estimated Ni^{2+} activity (nmol/L)	Relative reproduction at Zn 20 µg/L (%)	$EC10_{Zndiss}$ (µg/L)	$EC50_{Zndiss}$ (µg/L)	β_{Zndiss}	$EC10_{Zn2+}$ (nmol/L)	$EC50_{Zn2+}$ (nmol/L)	β_{Zn2+}
Natural DOC	**Ni 0**	<DL	<DL	0.9±0.4	0.7±0.5	2.32	100	133 (113-156)	280 (260-301)	2.97	598 (490-729)	1496 (1369-1634)	2.42
	Ni 18	15±4	14±4	14±1	14±2	93	103	184 (91-372)	241 (189-308)	8.18	893 (381-2091)	1247 (928-1677)	6.57
	Ni 32	25±7	25±7	24±2	25±3	179	94	159 (82-305)	249 (197-316)	4.85	745 (333-1667)	1301 (973-1739)	3.94
	Ni 56	45±13	45±6	43±3	44±7	362	101	70 (34-142)	187 (136-258)	2.22	270 (112-649)	912 (614-1353)	1.81
	Ni 100	82±18	81±18	79±9	80±7	649	71	83 (49-142)	132 (102-169)	4.81	332 (171-645)	589 (431-806)	3.83
	Ni 180	170±39	166±37	160±7	163±10	1502	32	22 (13-36)	37 (27-51)	4.11	69 (39-122)	128 (88-187)	3.54
	Ni 320	284±62	277±60	264±11	270±13	2556	3	-	-	-	-	-	
Aldrich Humic Acid	**Ni 0**	<DL	<DL	<DL	<DL	1.93	100	89 (40-199)	264 (139-480)	2.11	609 (294-1262)	2081 (1454-2819)	1.96
	Ni 10	8±2	7±2	5±1	6±1	59	116	67 (56-81)	265 (243-289)	1.60	438 (255-753)	1382 (1080-1767)	1.91
	Ni 18	14±3	13±3	9±2	11±3	107	91	116 (76-176)	207 (170-251)	3.79	710 (458-1100)	1477 (1215-1796)	3.00
	Ni 32	24±6	22±5	16±3	19±4	188	127	58 (37-90)	147 (118-182)	2.35	316 (191-523)	1070 (853-1342)	1.80
	Ni 56	71±19	64±18	54±10	59±12	589	99	120 (85-170)	173 (139-215)	6.03	811 (543-1211)	1295 (1054-1591)	4.69
	Ni 100	111±26	98±23	84±12	91±13	913	98	80 (45-143)	15 (115-208)	3.35	494 (269-940)	1101 (822-1475)	2.74
	Ni 180	180±40	165±37	140±16	152±18	1527	63	49 (22-107)	112 (79-159)	2.63	273 (120-620)	754 (522-1089)	2.16

DL_{Ni}=0.5 µg/L

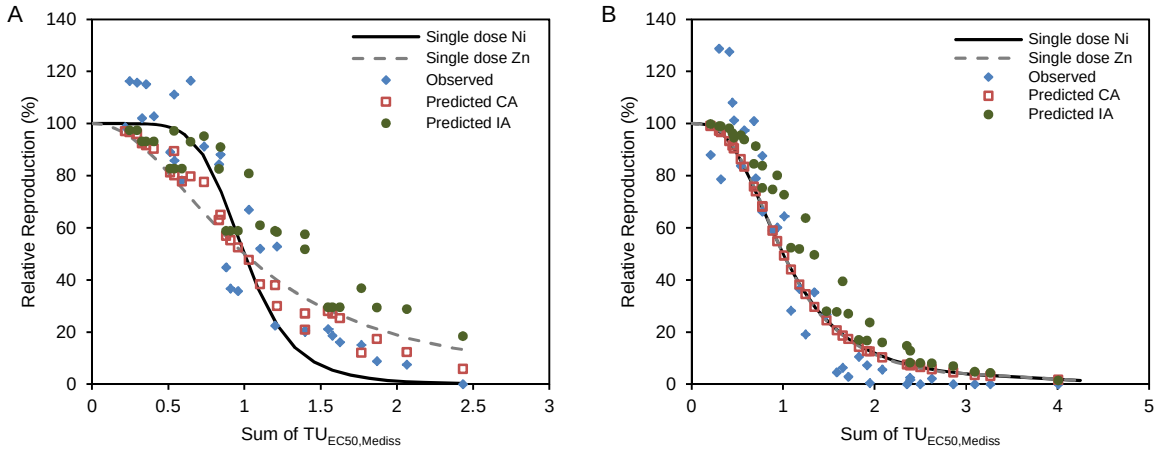

Figure B.2: Observed and predicted 21d-relative reproduction (RR) of *Daphnia magna* in the mixture combinations of the Ni-Zn mixture in function of the sum of Toxic Units (TU, expressed relative to EC50) based on dissolved Ni and Zn concentrations for the AHA-test series (A) and the natural DOC test series (B). Symbols are denoted as follows: observed effects (♦), predictions of CA (Eq. 5.4; □), predictions of IA (Eq. 5.5; ●). Predictions are based on the parameters ($EC50_{Mediss}$ and β_{Mediss}) of the individual concentration response curves of Ni and Zn (Eq. 5.3).

Table B.3. Estimated model parameters for the mixture reference models Concentration Addition (CA) and Independent Action (IA)[a] fitted to the binary Ni-Zn mixture (21d-)reproductive toxicity data (single metal treatments & mixture data) of *Daphnia magna*

		CA-non interaction		CA mixture interactions		IA-non interaction		IA mixture interactions	
		Ni	Zn	Ni	Zn	Ni	Zn	Ni	Zn
Natural DOC	$EC50_{Mediss}$ (µg/L)	123	284	131	216	94	239	105	261
	β_{Mediss}	5.37	3.94	5.03	3.16	3.16	3.65	3.32	4.06
	a^b	-		-0.00036		-		-2.25	
	AIC	375		406		388		383	
	SSE	5591		10357		7355		6424	
	F-test	-19.8 (p>0.99)				6.23 (p=0.02)			
	$EC50_{Me2+}$ (nmol/L)	943	1537	1098	1615	783	1266	993	1713
	β_{Me2+}	3.37	3.82	3.92	3.59	3.16	2.70	3.46	3.49
	a^b	-		-0.24		-		-3.41	
	AIC	375		378		385		382	
	SSE	5605		5752		6884		6209	
	F-test	-1.1 (p>0.99)				4.68 (p=0.04)			
Aldrich Humic Acid	$EC50_{Mediss}$ (µg/L)	212	245	192	233	150	193	163	213
	β_{Mediss}	9.06	3.60	11.01	2.74	5.25	3.22	7.19	3.80
	a^b	-		0.56		-		-2.36	
	AIC	324		324		327		323	
	SSE	5032		4884		5447		4697	
	F-test	1.09 (p=0.3)				5.74 (p=0.02)			
	$EC50_{Me2+}$ (nmol/L)	1964	1918	1963	1872	1472	1537	1609	1835
	β_{Me2+}	7.88	3.08	9.37	2.68	5.75	2.98	10.35	2.99
	a^b	-		0.21		-		-2.61	
	AIC	321		324		327		323	
	SSE	4766		4789		5438		4670	
	F-test	-0.17 (p>0.99)				5.91 (p=0.02)			

[a]Fitted using the mixture analysis framework of Jonker et al. (2005); EC50= effective concentration causing 50% effect, a=deviation parameter to quantify mixture interactions, AIC=akaike information criterion, SSE= sum of squared errors The F-test compares the nested models CA-non interaction with CA-mixture interaction and the IA-non-interaction with the IA-mixture interaction; $p < 0.05$ indicates significant deviation

[b] If $a<0$ the mixture components interact synergistic, if $a>0$ the mixture components interact antagonistic

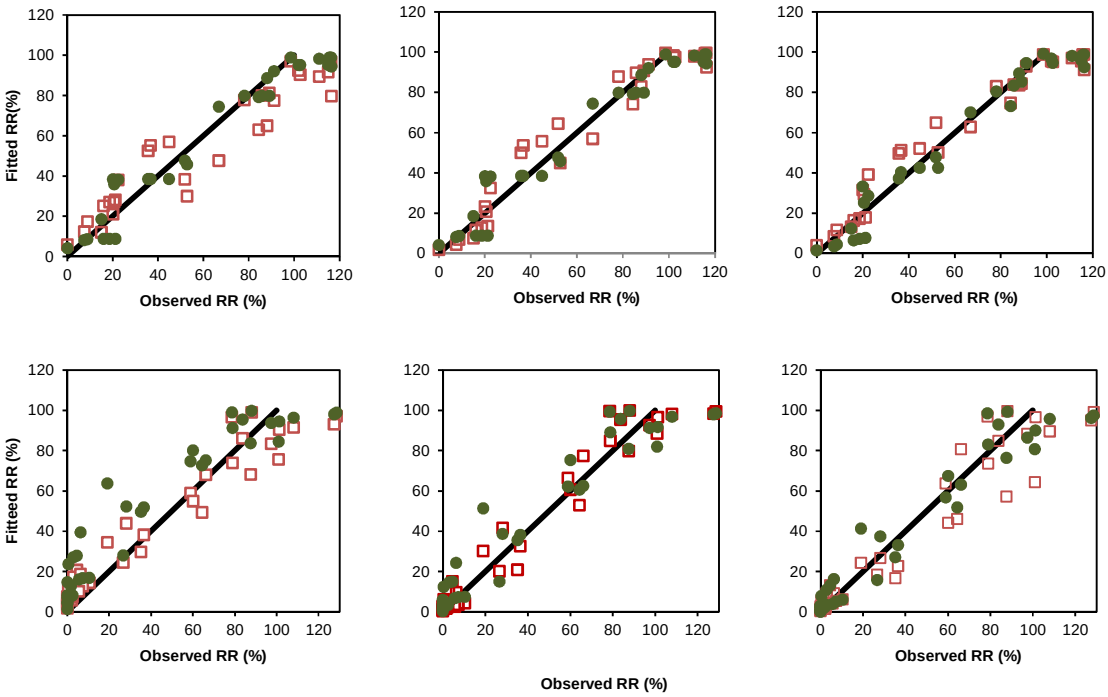

Figure B.3. Observed versus fitted 21d-relative reproduction (RR) of *Daphnia magna* for mixture reference models CA (squares) and IA (circles) after exposure to binary mixtures of Ni and Zn (only mixture data is shown) for the AHA- (upper panels) and the Natural DOC (lower panels) test series. Model parameters were derived based on dissolved concentrations. Left model fittings based on parameters of the single metal exposures, middle models fitted to all data (single metal and mixture treatments), right model extended with interactive effects parameter fitted to all data.

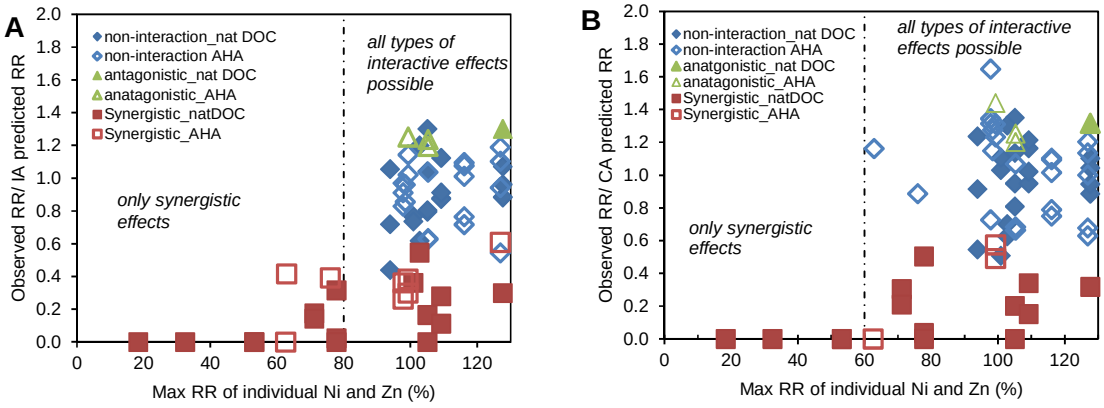

Figure B.4: Ratio of observed and IA (A) and CA (B) predicted 21d-relative reproduction (RR) of *Daphnia magna* for the mixture treatments in function of the maximum RR (%) in the corresponding single dose treatments of Ni and Zn. Values higher than 1 indicate possible antagonistic interactions, values lower than 1 indicate possible synergistic interactions. Symbols are denoted as follows: significant antagonistic interactions (triangles), significant synergistic interactions (squares) and non-interaction (predictions not significantly different from observed response, diamonds). Filled symbols denote treatments from the natural DOC series, open symbols from the AHA test series

Table B.4: Mean observed relative reproduction (RR; %) of *Daphnia magna* during 21d exposure to combinations of Ni and Zn for the Natural DOC test series. 95% confidence intervals on RR are reported between brackets. Predicted RR based on dissolved concentrations (IA/CA) are reported in italics.

	Zn 18 µg/L	Zn 32 µg/L	Zn 56 µg/L	Zn 100 µg/L	Zn 180 µg/L	Zn 320 µg/L	Zn 560 µg/L
Ni 0 µg/L	100 (91-109)	128 (108-148)	105 (60-150)	109 (91-128)	78 (40 112)	53 (32-75)	18 (1-35)
Ni 32 µg/L	103 (74-131)	88 (62-114) *100/99 (0/0)*[a]	79 (33-124) *99/97 (0/0)*	108 (88-128) *96/92 (0/0)*	101 (79-123) *84/76 (0/-)*	28 (6-50) *52/44 (+/0)*	10 (0-27) *17/14 (0/0)*
Ni 56 µg/L	94 (62-126)	129 (107-150) *99/97 (-/-)*	127 (93-162) *98/93 (0/0)*	84 (44-123) *95/86 (0/0)*	88 (50-125) *84/68 (0/0)*	37 (12-61) *52/38 (0/0)*	7 (2-17) *17/13 (0/0)*
Ni 100 µg/L	101 (62-140)	101 (59-144) *95/90 (0/0)*	97 (73-121) *94/83 (0/0)*	79 (40-118) *91/74 (0/0)*	60 (30-90) *80/55 (0/0)*	*35 (17-54)* 50/30 (0/0)	6 (0-12) *16/10 (+/0)*
Ni 180 µg/L	71 (46-96)	66 (30-102) *75/68 (0/0)*	59 (19-99) *75/59 (0/0)*	64 (49-79) *73/49 (0/0)*	20 (0-42) *64/35 (+/0)*	*6 (0-16)* 39/19 (+/+)	2 (0-5) *13/7 (+/+)*
Ni 320 µg/L	32 (15-50)	27 (12-42) *28/24 (0/0)*	4 (0-13) *28/21 (+/+)*	3 (0-9) *27/17 (+/+)*	0 (0-1) *24/12 (+/+)*	0 *15/8 (+/+)*	0 *5/3 (+/+)*
Ni 560 µg/L	3 (0-6)	2 (0-6) *8/7 (+/+)*	0 *8/7 (+/+)*	2 (2-6) *8/6 (+/0)*	0 *7/4 (+/+)*	0 *4/3 (+/+)*	0 *1/2 (+/+)*

[a] Deviations from non-interaction are reported between brackets; 0 non-interaction, + observed joint effect higher than predicted, - observed joint effect lower than predicted

Table B.5: Mean observed relative reproduction (RR; %) of *Daphnia magna* during 21d exposure to combinations of Ni and Zn for the AHA test series. 95% confidence intervals on RR are reported between brackets. Predicted RR based on dissolved concentrations (IA/CA) are reported in italics.

	Zn 18 µg/L	Zn 56 µg/L	Zn 100 µg/L	Zn 180 µg/L	Zn 320 µg/L	Zn 560 µg/L
Ni 0 µg/L	100 (82-118)	98 (71-126)	98 (62-133)	76 (51-101)	63 (48-78)	28 (12-44)
Ni 32 µg/L	116 (95-137)	99 (71-126) *97/97 (0/0)*[a]	102 (69-135) *93/92 (0/0)*	89 (77-101) *83/81 (0/0)*	45 (22-67) *59/57 (0/0)*	21 (5-37) *29/28 (0/0)*
Ni 56 µg/L	105 (83-127)	116 (98-134) 97/97 (-/-)	115 (100-130) *93/92 (-/-)*	86 (63-109) *83/80 (0/0)*	37 (13-60) *59/55 (0/0)*	19 (1-36) *29/27 (0/0)*
Ni 100 µg/L	127 (102-152)	116 (95-136) *97/96 (0/0)*	103 (83-123) *93/90 (0/0)*	78 (47-109) *83/78 (0/0)*	36 (16-55) *59/52 (+/0)*	16 (0-32) *29/25 (0/0)*
Ni 180 µg/L	99 (71-128)	111 (85-137) *97/89 (0/0)*	116 (97-135) *93/80 (-/-)*	84 (59-110) *83/63 (0/0)*	22 (6-39) *59/38 (+/0)*	9 (1-16) *29/17 (+/+)*
Ni 320 µg/L	98 (76-120)	91 (48-134) *95/78 (0/0)*	88 (54-122) *91/65 (0/0)*	67 (43-91) *81/48 (0/0)*	21 (0-41) *57/27 (+/0)*	8 (0-17) *29/12 (+/0)*
Ni 560 µg/L	63 (39-86)	52 (24-80) *61/38 (0/0)*	53 (43-91) *58/30 (0/0)*	20 (2-38) *52/21 (+/0)*	15 (1-29) *37/12 (+/0)*	0 *18/6 (+/+)*

[a] Deviations from non-interaction are reported between brackets; 0 non-interaction, + observed joint effect higher than predicted, - observed joint effect lower than predicted

Appendix C: Chapter 6: Reproductive toxicity of binary and ternary mixture combinations of Ni, Zn, and Pb to *Ceriodaphnia dubia* is best predicted with the independent action model

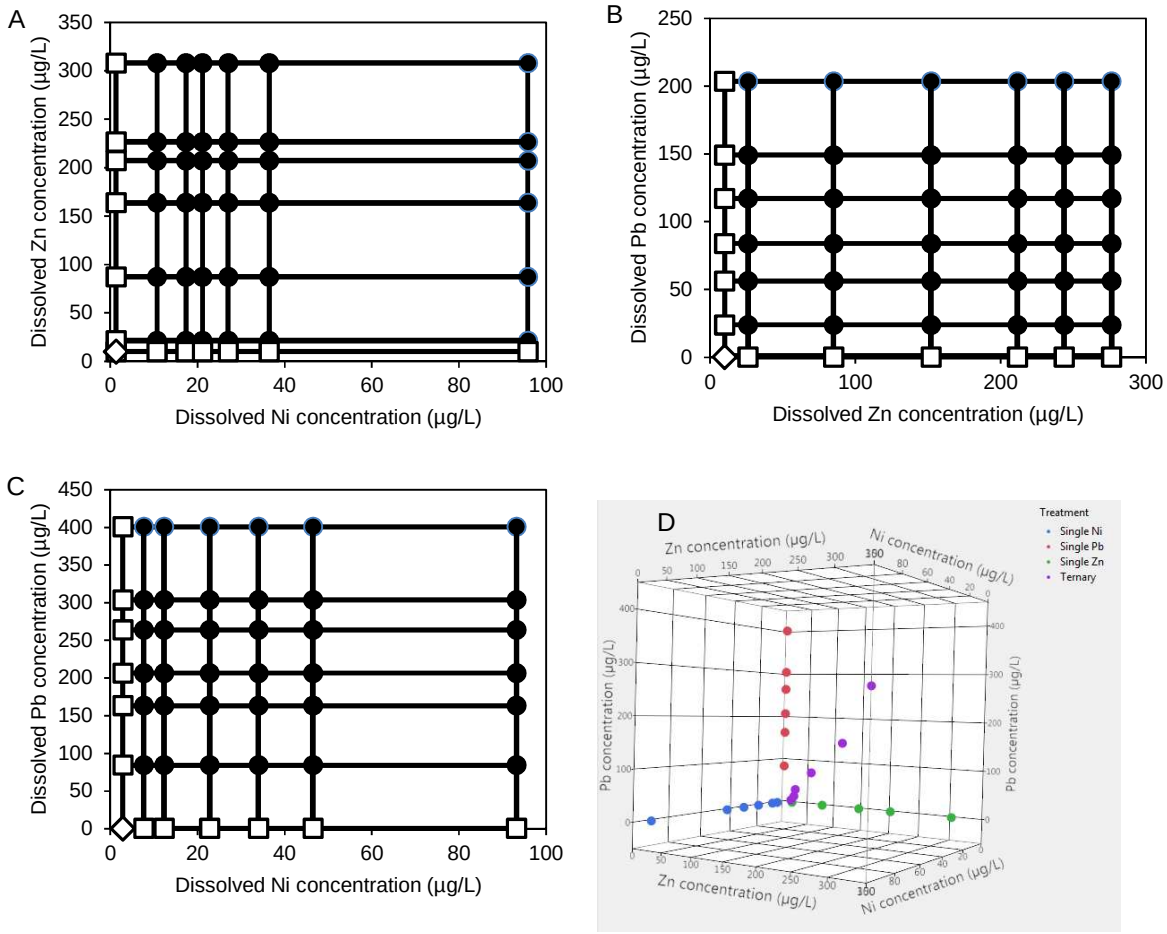

Figure C.1 Test design investigating the 7d-reproductive toxicity of the binary Ni-Zn mixture (A), Pb-Zn mixture (B), and Ni-Pb mixture (C) and Ni-Pb-Zn mixture (D) to *Ceriodaphnia dubia*. In plot A-C, concentrations are the average of measured dissolved concentrations in new and old media. Filled symbols indicate mixture treatments of Ni, Zn, and/or Pb. Open squares indicate individual Ni, Zn, or Pb toxicity treatments (mixture control). The diamond indicates the absolute control treatments (no Ni, Zn, or Pb added). In Plot D, blue, red and green dots represent the individual metal treatments of Ni, Pb, and Zn. Purple dots represent the ternary metal mixture treatments (investigated at 0.1, 0.2, 0.5, 1.0, 2.0 and 3.0 ΣTU_{Mix}). Toxicity of the Ni-Zn-Pb mixture was tested concurrently with the binary full-factorial Pb-Ni test (plot C).

Table C.1 Control performance of the *Ceriodaphnia dubia* reproduction tests relative to the validation criteria described in the USEPA (2002a) test protocol. The results of the Kruskal-Wallis test for inter-plate differences in control reproduction is also listed.

Test id	Number of control replicates	Mean control reproduction (± standard error)[a]	Control mortality (%)	Mothers with 3 broods (%)[c]	Kruskal-Wallis test for inter-plate differences in control reproduction
Expt. 1: Ni-Zn	78	15.3±0.9	5	71	χ^2=2.62, p=0.92
Expt. 2: Pb-Zn	73	20.5±0.9	1	85	χ^2=7.19, p=0.41
Expt. 3: Ni-Pb & Ni-Pb-Zn	91	17.3±0.7	4	76	χ^2=6.17, p=0.72

[a] Control organisms should produce on average at least 15 juveniles (USEPA, 2002a)
[b] Control mortality should be less than 20% (USEPA, 2002a)
[c] At least 60% of the control organisms should have had 3 broods (USEPA, 2002a)

Table C.2. 10%, 20% and 50% 7d-effective concentrations (EC10, EC20, and EC50, respectively, using relative reproduction as endpoint) of *Ceriodaphnia dubia* in the individual Zn, Ni and Pb exposures in Expts. 1, 2 and 3.

Expt. ID	Parameter	Zn_{diss} [a]	Ni_{diss} [a]	Pb_{diss} [a]	Zn^{2+} [b]	Ni^{2+} [b]	Pb^{2+} [b]
Expt. 1: Ni-Zn	**EC50**	234 (212-257)	43 (28-66)	-	2014 (1813-2238)	478 (295-774)	-
	EC20	203 (180-230)	33 (25-43)	-	1731 (1516-1978)	361 (271-479)	-
	EC10	187 (154-227)	28 (18-44)	-	1585 (1284-1958)	306 (185-507)	-
Expt. 2: Pb-Zn	**EC50**	239 (223-256)	-	111 (101-122)	1993 (1849-2148)	-	20 (18-23)
	EC20	211 (186-238)	-	91 (77-107)	1733 (1514-1984)	-	15 (13-19)
	EC10	195 (164-233)	-	81 (65-100)	1597 (1320-1932)	-	13 (10-17)
Expt. 3: Ni-Pb & Ni-Pb-Zn	**EC50**	197 (165-236)	52 (41-66)	302 (290-314)	1290 (1050-1584)	546 (422-706)	36 (34-39)
	EC20	134 (97-186)	31 (23-43)	282 (256-310)	832 (576-1202)	315 (226-439)	33 (29-38)
	EC10	108 (68-171)	23 (15-36)	271 (235-312)	644 (380-1091)	228 (142-367)	31 (25-38)

[a] Dissolved concentrations are reported in µg/L
[b] Free ion activities are reported in nmol/L

Background metal concentrations in the Brisy natural water

On both sampling occasions natural background Zn, Pb and Cd concentrations (measured as dissolved concentrations) were below detection limit (see section *Analytical chemistry*). Background Ni concentrations were 1.3 and 2.8 µg dissolved Ni/L for sampling occasion 1 (Expt. 1 & 2) and 2 (Expt. 3), respectively. For Cu, background concentrations were on both sampling occasions 2 µg dissolved Cu/L.

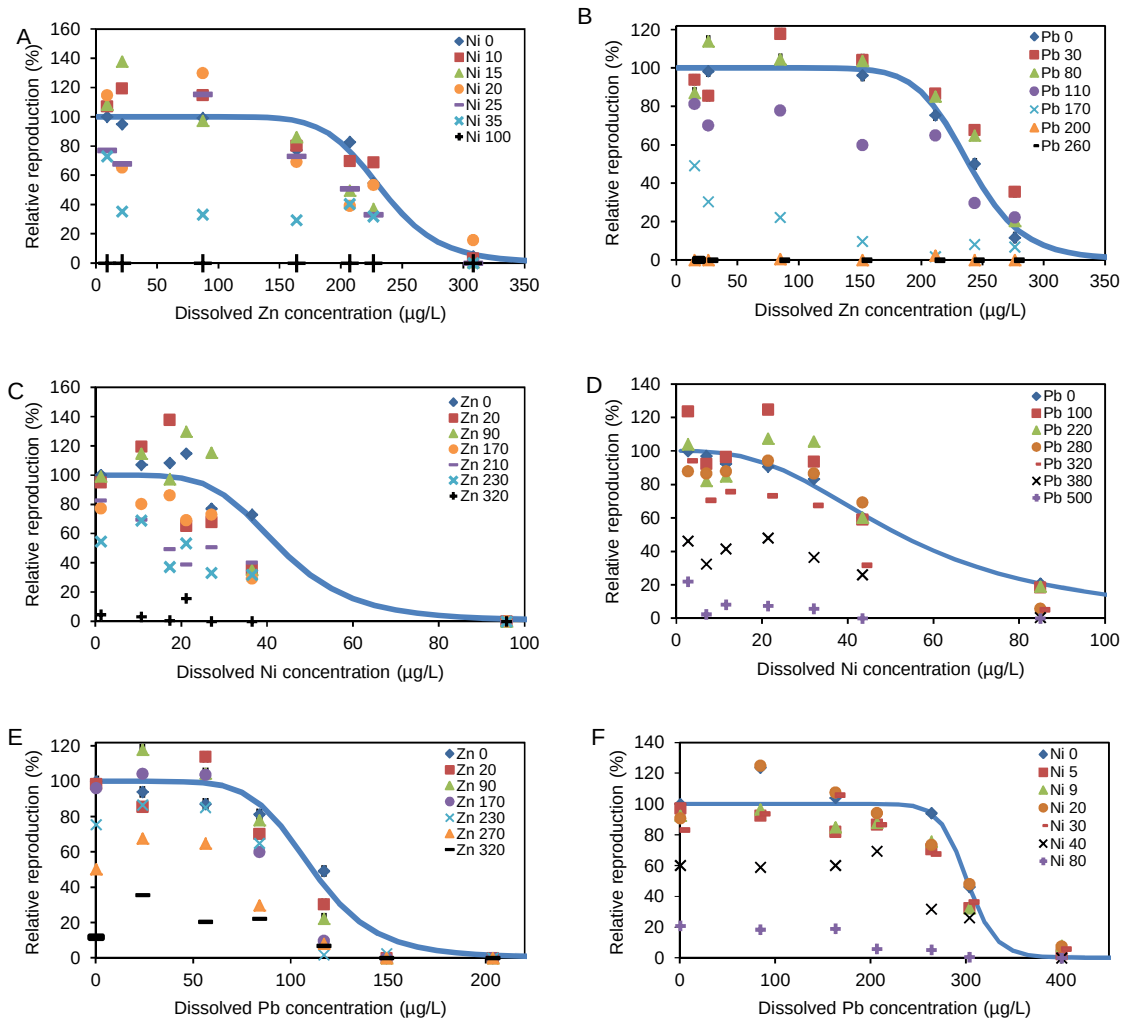

Figure C.2. Dose- response data for 7d-reproduction relative to the control (no metals added; symbols) for *Ceriodaphnia dubia* as a function of dissolved metal concentrations in binary mixtures of Ni, Zn, and Pb. A & C are data of Expt. 1 (Ni-Zn mixture), B& E of Expt. 2 (Pb-Zn mixture) and D & F of Expt. 3 (Ni-Pb mixture). Lines represent the fitted log-logistic dose-response curves of the individual metal exposures (Equation 6.4). Parameters of the dose-response curves are reported in Table 6.2. Standard errors are reported in Table C.3-5.

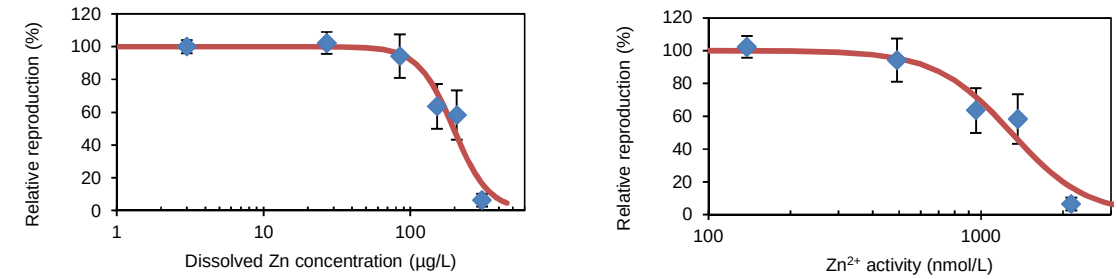

Figure C.3 Concentration response data (symbols, based on 7d-reproduction) of *Ceriodaphnia dubia* for the single Zn exposure treatments in the Ni-Pb-Zn experiment based on dissolved Zn concentration (left panel) and free ion activities (right panel). Error bars indicate standard errors (n=10). Lines are the fitted log-logistic concentration response curves (Equation 6.4), parameters of the concentration-response curves are reported in Table 6.2.

Table C.3 Measured dissolved concentrations, WHAM VII calculated free ion activities, average and standard error on number of juveniles, average relative reproduction and standard error on relative response in the 7d days exposure to Ni-Zn mixtures with *Ceriodaphnia dubia*.

treatment nr	Dissolved concentrations (µg/L)		Free ion activitiess (nmol/L)		Number of juveniles		Relative response (%)	
	Zn	Ni	Zn^{2+}	Ni^{2+}	Average	St. error	Average	St. error
1	10	1.3	64	13	15.3	0.9	100	6
2	21	1.3	156	14	14.6	1.6	95	10
3	87	1.3	694	14	15.2	2.7	99	18
4	164	1.3	1370	15	11.9	1.5	77	9
5	207	1.3	1770	15	12.7	2.5	83	17
6	227	1.3	1950	15	8.4	1.9	55	12
7	308	1.3	2719	15	0.7	0.4	5	3
8	10	11	66	115	16.4	2.8	107	18
15	10	17	66	188	16.6	2.5	108	16
22	10	21	67	230	17.6	2.3	115	15
29	10	27	67	296	11.8	2.4	77	15
36	10	36	68	402	11.2	1.4	73	9
43	10	96	70	1081	0.0	0.0	0	0
9	21	11	157	117	18.3	2.5	119	17
10	87	11	697	121	17.6	2.5	115	17
11	164	11	1373	124	12.3	2.3	80	15
12	207	11	1772	125	10.7	2.7	70	18
13	227	11	1952	125	10.6	1.8	69	12
14	308	11	2723	126	0.5	0.5	3	3
16	21	17	159	190	21.1	2.6	138	17
17	87	17	699	197	14.9	2.0	97	13
18	164	17	1375	201	13.2	2.4	86	15
19	207	17	1774	203	7.6	2.5	50	16
20	227	17	1952	203	5.7	1.7	37	11
21	308	17	2724	205	0.1	0.1	1	1
23	21	21	159	233	10.0	1.9	65	13
24	87	21	700	241	19.9	2.0	130	13
25	164	21	1376	245	10.6	2.0	69	13
26	207	21	1776	247	6.0	1.3	39	9
27	227	21	1954	248	8.2	2.1	53	14
28	308	21	2725	250	2.4	1.0	16	7
30	21	27	160	299	10.4	2.2	68	14
31	87	27	701	308	17.7	1.5	115	10
32	164	27	1378	314	11.2	2.2	73	15
33	207	27	1777	316	7.8	2.2	51	14
34	227	27	1957	317	5.1	1.3	33	9
35	308	27	2727	320	0.0	0.0	0	0
37	21	36	161	405	5.4	1.0	35	7
38	87	36	704	417	5.1	1.9	33	12
39	164	36	1380	424	4.5	1.9	29	12
40	207	36	1780	427	6.2	2.0	40	13
41	227	36	1958	428	4.9	1.8	32	12
42	308	36	2730	432	0.0	0.0	0	0
44	21	96	166	1087	0.0	0.0	0	0
45	87	96	715	1107	0.0	0.0	0	0
46	164	96	1394	1122	0.0	0.0	0	0
47	207	96	1794	1128	0.0	0.0	0	0
48	227	96	1973	1130	0.0	0.0	0	0
49	308	96	2487	904	0.0	0.0	0	0

DL_{Zn}=10 µg/L

Table C.4 Measured dissolved concentrations, WHAM VII calculated free ion cactivities, average and standard error on number of juveniles, average relative response and standard error on relative response in the 7d days exposure to Zn-Pb mixtures with *Ceriodaphnia dubia*.

treatment number	Dissolved concentrations (µg/L)		Free metal activities (nmol/L)		Number of juveniles		Relative response (%)	
	Zn	Pb	Zn^{2+}	Pb^{2+}	Average	St. error	Average	St. error
1	10	<DL[a]	66	0.03	20.5	0.9	100	5
2	26	<DL	180	0.03	20.2	1.8	98	9
4	152	<DL	1212	0.04	19.8	4.6	96	23
5	212	<DL	1743	0.05	15.5	2.2	75	11
6	244	<DL	2036	0.05	10.3	2.7	50	13
7	276	<DL	2339	0.05	2.4	1.6	12	8
8	10	24	68	3.28	19.3	2.4	94	12
15	10	56	70	8.61	17.9	2.3	87	11
22	10	84	72	14.04	16.7	1.8	81	9
29	10	117	74	21.57	10.1	2.0	49	10
36	10	149	76	29.94	0.0	0.0	0	0
43	10	204	79	46.62	0.0	0.0	0	0
9	26	24	184	3.46	17.6	2.5	85	12
10	84	24	651	4.01	24.2	0.8	118	4
11	152	24	1228	4.53	21.4	2.0	104	10
12	212	24	1761	4.95	17.8	1.4	87	7
13	244	24	2056	5.16	13.9	2.1	68	10
14	276	24	2360	5.37	7.3	1.8	36	9
16	26	56	189	9.04	23.4	1.2	114	6
17	84	56	664	10.36	21.4	1.7	104	8
18	152	56	1247	11.60	21.3	2.8	104	14
19	212	56	1784	12.58	17.5	2.2	85	11
20	244	56	2081	13.08	13.3	2.1	65	10
21	276	56	2388	13.57	4.2	2.1	20	10
23	26	84	194	14.68	14.4	2.4	70	12
24	84	84	675	16.65	16.0	2.1	78	10
25	152	84	1263	18.51	12.3	1.8	60	9
26	212	84	1804	19.97	13.3	1.7	65	8
27	244	84	2102	20.72	6.1	2.2	30	11
28	276	84	2409	21.45	4.6	1.9	22	9
30	26	117	199	22.45	6.2	2.5	30	12
31	84	117	688	25.18	4.6	1.7	22	8
32	152	117	1281	27.77	2.0	1.0	10	5
33	212	117	1825	29.83	0.4	0.4	2	2
34	244	117	2125	30.87	1.7	1.3	8	6
35	276	117	2434	31.90	1.4	1.0	7	5
37	26	149	203	31.04	0.0	0.0	0	0
38	84	149	699	34.48	0.1	0.1	0	0
39	152	149	1297	37.79	0.0	0.0	0	0
40	212	149	1845	40.42	0.5	0.5	2	2
41	244	149	2147	41.76	0.0	0.0	0	0
42	276	149	2458	43.08	0.0	0.0	0	0
44	26	204	210	48.07	0.0	0.0	0	0
45	84	204	717	52.69	0.0	0.0	0	0
46	152	204	1323	57.19	0.0	0.0	0	0
47	212	204	1878	60.80	0.0	0.0	0	0
48	244	204	2182	62.64	0.0	0.0	0	0
49	276	204	2365	58.91	0.0	0.0	0	0

[a]When measured concentrations were below the limit of quantification (LOQ), the limit of quantification divided by two was used as the concentration input for further analyses: LOQ_{Pb}=1.4 µg/L

Table C.5 Measured dissolved concentrations, WHAM VII calculated free ion activities, average and standard error on number of juveniles, average relative response and standard error on relative response in the Ni-Pb & Ni-Zn-Pb experiment with *Ceriodaphnia dubia* (using 7d-reproductive toxicity as endpoint).

treatment nr	Dissolved conc. (µg/L)			Free ion activities (nmol/L)			Number of juveniles		Relative response (%)	
	Zn	Ni	Pb	Zn^{2+}	Ni^{2+}	Pb^{2+}	Average	St. error	Average	St. error
1	10	2.8	<DL[a]	-	25	0.02	17.3	0.7	100	4
2	-	8	<DL	-	68	0.02	16.8	2.4	97	14
3	-	12	<DL	-	111	0.02	16.0	2.4	92	14
4	-	23	<DL	-	211	0.02	15.7	1.1	91	6
5	-	34	<DL	-	321	0.02	14.4	2.2	83	13
6	-	46	<DL	-	447	0.02	10.4	1.7	60	10
7	-	93	<DL	-	932	0.02	3.6	0.8	21	5
8	-	2.8	84	-	26	6.78	21.4	1.6	124	9
15	-	2.8	163	-	27	15.43	18.0	2.9	104	17
22	-	2.8	207	-	27	21.19	15.2	1.9	88	11
29	-	2.8	264	-	28	29.89	16.3	1.2	94	7
36	-	2.8	304	-	28	36.71	8.0	1.8	46	10
43	-	2.8	401	-	29	55.85	0.1	0.1	1	1
50	27	-	-	138	-	-	17.7	1.2	102	7
51	85	-	-	492	-	-	16.3	2.3	94	13
52	152	-	-	958	-	-	11.0	2.4	64	14
54	29	-	-	1365	-	-	10.1	2.6	58	15
55	207	-	-	2145	-	-	1.1	0.7	6	4
9	-	8	84	-	71	6.83	16.0	2.3	92	13
10	-	12	84	-	115	6.87	16.7	2.3	96	13
11	-	23	84	-	218	6.97	21.6	1.7	125	10
12	-	34	84	-	331	7.06	16.2	2.0	94	12
13	-	46	84	-	459	7.16	10.2	1.8	59	11
14	-	93	84	-	952	7.48	3.2	0.6	18	4
16	-	8	163	-	73	15.53	14.2	1.9	82	11
17	-	12	163	-	118	15.62	14.7	1.8	85	10
18	-	23	163	-	224	15.80	18.6	1.1	107	7
19	-	34	163	-	339	15.99	18.3	1.7	106	10
20	-	46	163	-	469	16.18	10.4	1.7	60	10
21	-	93	163	-	968	16.80	3.3	0.8	19	4
23	-	8	207	-	74	21.31	15.0	2.0	87	12
24	-	12	207	-	120	21.42	15.2	2.7	88	15
25	-	23	207	-	227	21.65	16.3	1.4	94	8
26	-	34	207	-	343	21.88	15.0	2.0	87	11
27	-	46	207	-	475	22.11	12.0	1.8	69	10
28	-	93	207	-	977	22.89	1.0	0.6	6	3
30	-	8	264	-	75	30.04	12.2	1.6	71	9
31	-	12	264	-	122	30.18	13.1	1.7	76	10
32	-	23	264	-	230	30.47	12.7	1.9	73	11
33	-	34	264	-	348	30.75	11.7	1.3	68	8
34	-	46	264	-	481	31.04	5.5	1.2	32	7
35	-	93	264	-	987	32.01	0.9	0.4	5	2
37	-	8	304	-	76	36.88	5.6	2.2	32	12
38	-	12	304	-	123	37.03	7.2	2.3	42	13
39	-	23	304	-	232	37.36	8.3	1.9	48	11
40	-	34	304	-	351	37.67	6.3	2.2	36	13
41	-	46	304	-	484	38.00	4.5	1.6	26	9
42	-	93	304	-	993	39.10	0.1	0.1	1	1
44	-	8	401	-	78	56.07	0.4	0.3	2	2
45	-	12	401	-	126	56.26	1.4	1.0	8	6
46	-	23	401	-	237	56.67	1.3	1.1	8	6
47	-	34	401	-	357	57.06	1.0	0.7	6	4
48	-	46	401	-	493	57.47	0.0	0.0	0	0
49	-	93	401	-	1007	58.83	0.0	0.0	0	0
56	27	4	6	138	36	0.47	21.6	2.7	125	16
57	35	5	16	188	49	1.24	17.0	2.1	98	12
58	42	7	33	235	71	2.68	19.8	2.1	114	12
59	76	9	75	462	95	7.15	16.6	1.1	96	6
60	160	23	149	1094	243	18.59	8.8	2.3	51	13
61	225	33	270	1664	367	42.39	0.8	0.6	5	4

[a]When measured concentrations were below the limit of quantification (LOQ), the limit of quantification divided by two was used as the concentration input for further analyses: LOQ_{Pb}=1.4 µg/L

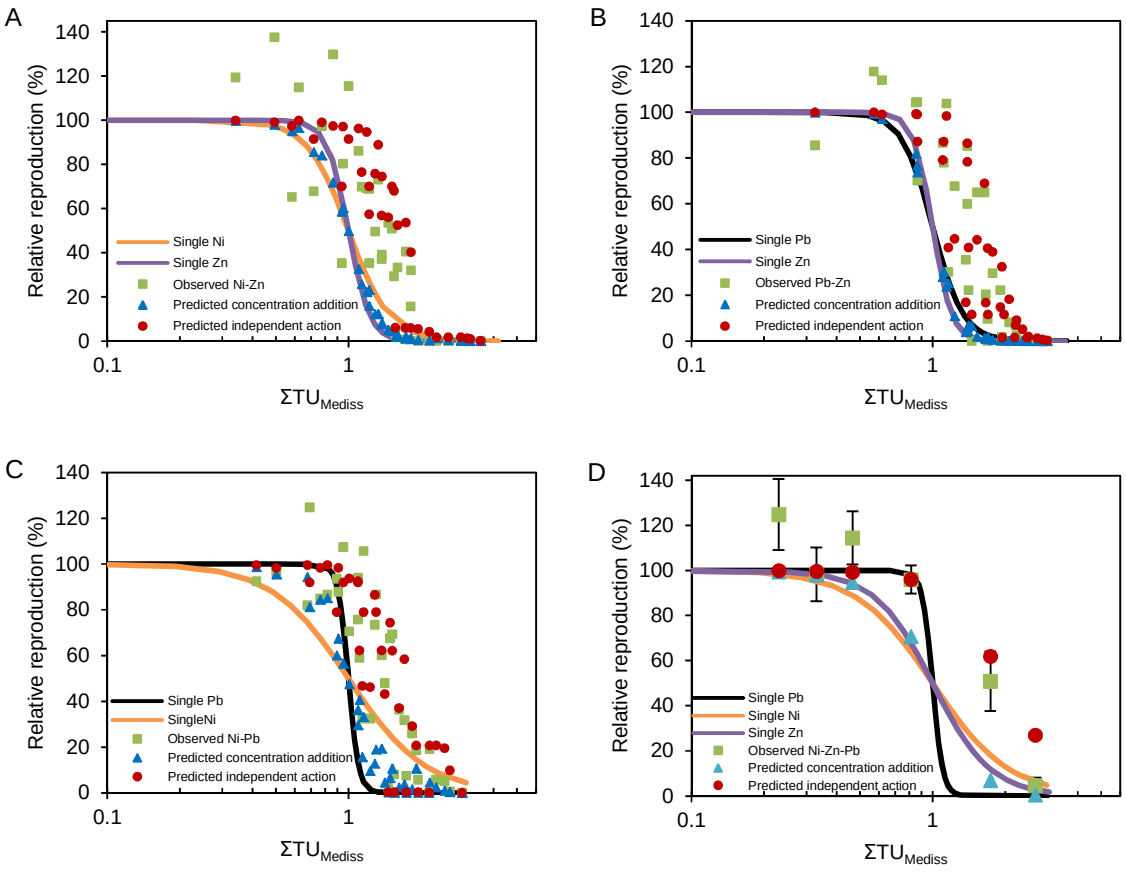

Figure C.4. Observed and predicted 7d-relative reproduction of *Ceriodaphnia dubia* in the binary mixture combinations of Ni, Zn, and Pb as a function of the sum of toxic units based dissolved concentrations (ΣTU_{Mediss}) for the Ni-Zn mixture (Expt. 1; A), Pb-Zn mixture (Expt. 2; B), the Ni-Pb mixture (Expt. 3; C), and the Ni-Pb-Zn mixture (Expt. 4; D). Symbols are denoted as follows: observed reproduction (squares), predictions of concentration addition (Equation 6.5, circles), and predictions of independent action (Equation 6.6, diamonds). Predictions are based on the parameters ($EC50_{Mediss,i}$ and $\beta_{Mediss,i}$)of the individual concentration-response curves of Ni, Zn, and Pb (Equation 6.4, lines).

Table C.6. Estimated model parameters for the mixture reference models Concentration Addition (CA; Equation 5) and Independent Action (IA; Equation 6)[a] fitted to the binary Ni-Zn mixture toxicity data (single metal treatments & mixture data) of *Ceriodaphnia dubia* (Expt. 1)

	CA non-interactive		CA interactive		IA non-interactive		IA interactive	
	Zn	**Ni**	**Zn**	**Ni**	**Zn**	**Ni**	**Zn**	**Ni**
$EC50_{Mediss}$ (µg/L)	287	60	242	37	220	35	233	36
β_{Mediss}	4.88	4.59	13.1	4.12	6.57	7.66	6.09	13.82
***a*[b]**	-		**1.57**		-		**-1.42**	
AIC	429		409		403		405	
F-test[c]	**F=24.3, p<0.001**				**F=-0.33, p=1.00**			
$EC50_{Me2+}$ (nmol/L)	2391	695	1966	508	1897	397	2003	448
β_{Me2+}	10.7	3.31	15.1	3.24	5.50	7.13	4.71	5.72
a	-		**1.63**		-		**-0.45**	
AIC	429		412		403		408	
F-test	**F=21.5, p<0.001**				**F=-2.5,p=1.00**			

[a] Fitted using the mixture analysis framework of Jonker et al. (2005) as described by Hochmuth et al. (2014);
[b] If $a<0$ the mixture components interact synergistically, if $a>0$ the mixture components interact antagonistically
[c] The F-test compares the nested models CA-non interaction with CA-mixture interaction and the IA-non-interaction with the IA-mixture interaction; $p < 0.05$ indicates significant deviation from non-interactivity. The type of interactive effect can be interpreted from the deviation parameter a[b]
EC50= effective concentration causing 50% effect, β=slope of the concentration response curve, *a*=deviation parameter to quantify mixture interactions, AIC=akaike information criterion

Table C.7. Estimated model parameters for the mixture reference models Concentration Addition (CA) and Independent Action (IA)[a] fitted to the binary Pb-Zn mixture toxicity data (single metal treatments & mixture data) of *Ceriodaphnia dubia* (Expt. 2)

	CA non-interactive		CA interactive		IA non-interactive		IA interactive	
	Pb	**Zn**	**Pb**	**Zn**	**Pb**	**Zn**	**Pb**	**Zn**
$EC50_{Mediss}$ (µg/L)	125	263	92	235	96	260	94	252
β_{Mediss}	7.28	1.39	8.13	12.04	5.18	17.11	8.1	17.41
***a*[b]**	-		**2.42**		-		**0.77**	
AIC	450		395		389		390	
F-test[c]	**F=100, p<0.001**				**F=1.09, p=0.30**			
$EC50_{Me2+}$ (nmol/L)	28	2502	19	1980	21	2151	18	2063
β_{Me2+}	7.15	4.23	5.10	10.3	6.13	8.56	4.84	8.93
a	-		**2.31**		-		**2.39**	
AIC	431		355		344		338	
F-test	**F=177, p<0.001**				**F=8.1, p=0.007**			

[a]Fitted using the mixture analysis framework of Jonker et al. (2005) as described by Hochmuth et al. (2014);
[b] If $a<0$ the mixture components interact synergistically, if $a>0$ the mixture components interact antagonistically
[c] The F-test compares the nested models CA-non interaction with CA-mixture interaction and the IA-non-interaction with the IA-mixture interaction; $p < 0.05$ indicates significant deviation from non-interactivity. The type of interactive effect can be interpreted from the deviation parameter a[b]
EC50= effective concentration causing 50% effect, β=slope of the concentration response curve, *a*=deviation parameter to quantify mixture interactions, AIC=akaike information criterion

Table C.8. Estimated model parameters for the mixture reference models Concentration Addition (CA) and Independent Action (IA)[a] fitted to the binary Ni-Pb mixture toxicity data (single metal treatments & mixture data) of *Ceriodaphnia dubia* (Expt. 3)

	CA non-interactive		CA interactive		IA non-interactive		IA interactive	
	Pb	**Ni**	**Pb**	**Ni**	**Pb**	**Ni**	**Pb**	**Ni**
$EC50_{Mediss}$ (µg/L)	328	79	281	53	290	52	293	48
β_{Mediss}	22.76	2.8	17.28	4.17	11.35	3.83	12.04	3.95
***a*[b]**	-		**1.91**		-		**0.30**	
AIC	431		373		364		366	
F-test[c]	**F=105.1, p<0.001**				**F=-0.20, p=1.00**			
$EC50_{Me2+}$ (nmol/L)	40	959	34	580	37	574	38	556
β_{Me2+}	7.39	2.28	13.6	3.31	5.83	3.69	6.65	3.76
a	-		**1.93**				**-0.56**	
AIC	408		372		365		370	
F-test	**F=53.2, p<0.001**				**F=-2.7, p=1.00**			

[a]Fitted using the mixture analysis framework of Jonker et al. (2005) as described by Hochmuth et al. (2014);
[b] If $a<0$ the mixture components interact synergistically, if $a>0$ the mixture components interact antagonistically
[c] The F-test compares the nested models CA-non interaction with CA-mixture interaction and the IA-non-interaction with the IA-mixture interaction; $p < 0.05$ indicates significant deviation from non-interactivity. The type of interactive effect can be interpreted from the deviation parameter a[b]
EC50= effective concentration causing 50% effect, β=slope of the concentration response curve, *a*=deviation parameter to quantify mixture interactions, AIC=akaike information criterion

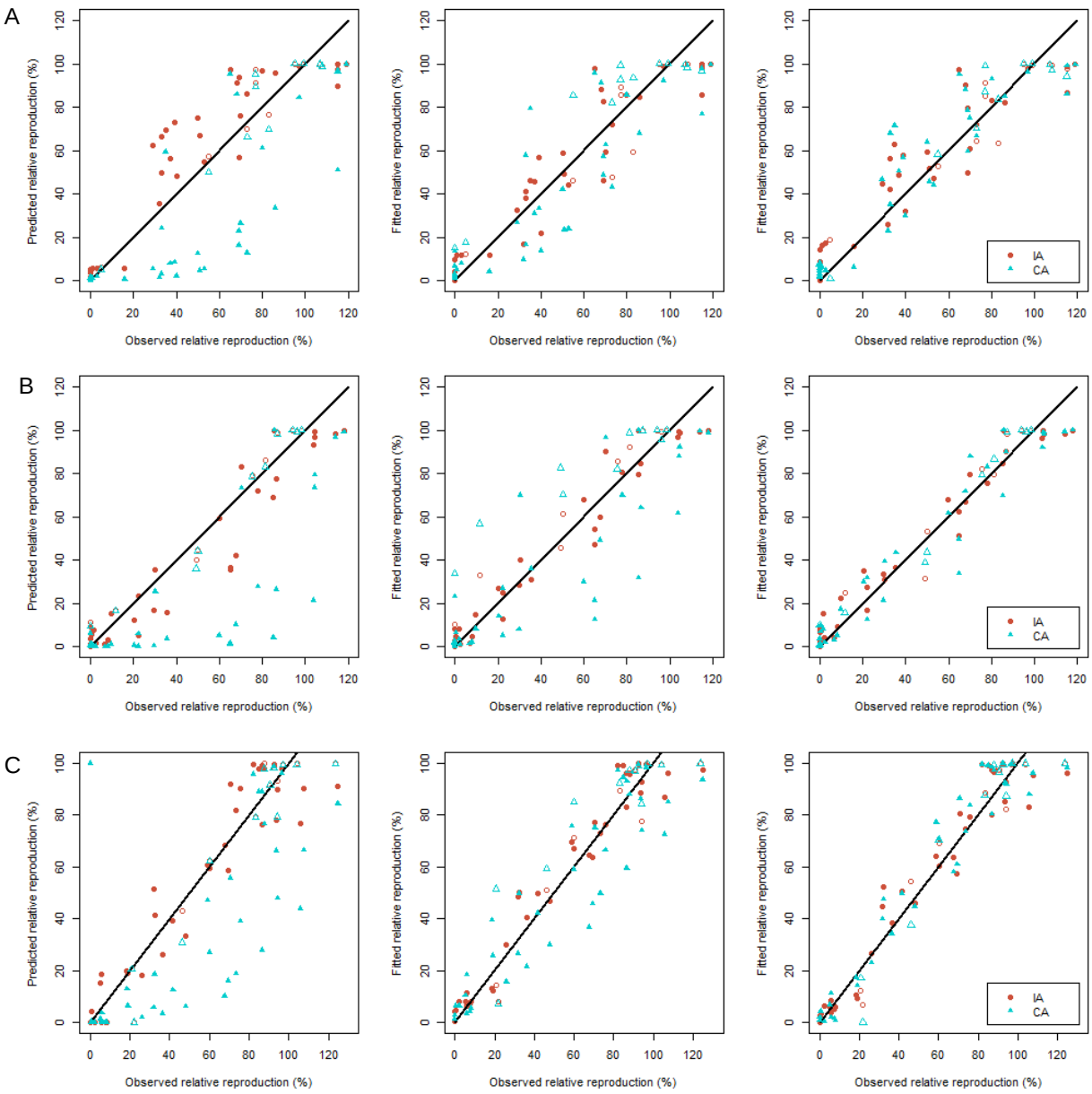

Figure C.5. Observed 7d-relative reproduction (%) of *Ceriodaphnia dubia* versus predicted or fitted relative reproduction (%) for the concentration addition (triangles) and independent action (circles) reference models after exposure to the binary Ni-Zn (Expt. 1; A), Pb-Zn (Expt. 2; B), and Ni-Pb (Expt. 3; C) mixtures. Model parameters were based on free ion activities. Left panel shows model predictions based on parameters estimated from the single-metal exposures alone, middle panels show models fitted to all data (single-metal and mixture treatments), right panel shows model extended with deviation parameter *a* fitted to all data. Open symbols represent data of single metal exposures, filled symbols represent data of mixture treatments.

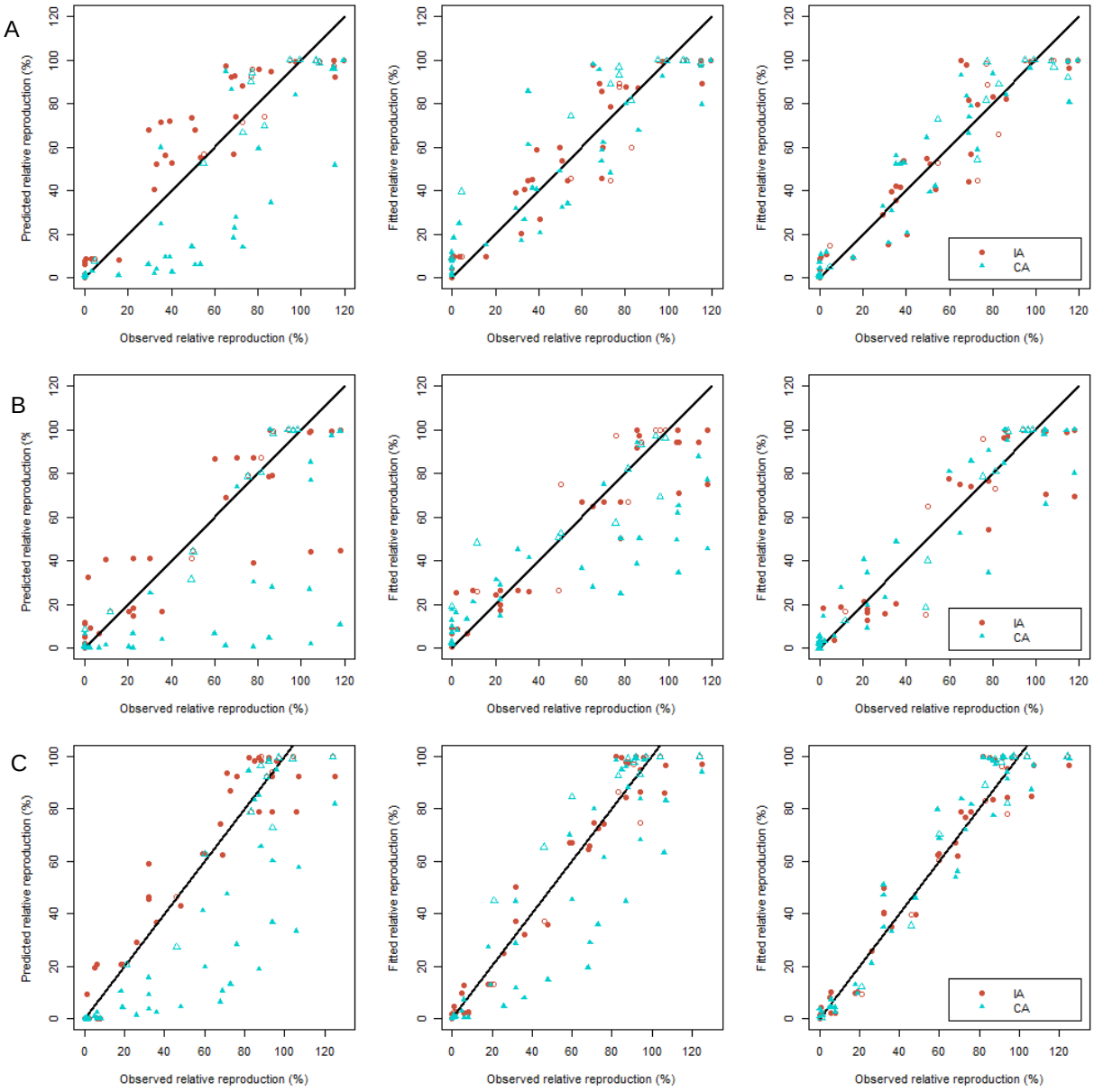

Figure C.6. Observed 7d-relative reproduction (%) of *Ceriodaphnia dubia* versus predicted or fitted relative reproduction (%) for the concentration addition (triangles) and independent action (circles) reference models after exposure to the binary Ni-Zn (Expt. 1; A), Pb-Zn (Expt. 2; B), and Ni-Pb (Expt. 3; C) mixtures. Model parameters were based on dissolved concentrations. Left panel shows model predictions based on parameters estimated from the single-metal exposures alone, middle panels show models fitted to all data (single-metal and mixture treatments), right panel shows model extended with deviation parameter *a* fitted to all data. Open symbols represent data of single metal exposures, filled symbols represent data of mixture treatments.

Table C.9 Estimated model parameters for the mixture reference models Concentration Addition (CA) and Independent Action (IA)[a] fitted to the ternary Ni-Zn-Pb mixture toxicity data (single metal treatments & mixture data) of *Ceriodaphnia dubia* (Expt. 3)

	CA non-interactive			CA interactive			IA non-interactive			IA interactive		
	Pb	**Ni**	**Zn**	**Pb**	**Ni**	**Zn**	**Pb**	**Ni**	**Zn**	**Pb**	**Ni**	**Zn**
$EC50_{Mediss}$ (µg/L)	331	57	239	323	56	225	300	54	187	297	52	184
β_{Mediss}	17.82	2.69	3.24	28.53	2.38	5.3	12.87	2.42	4.39	20.99	2.82	3.95
***a*[b]**		-			**9.31**			-			**-10.31**	
AIC		199			195.8			187.08			187.3	
F-test[c]	**F=10.8, p=0.005**						**F=1.29, p=0.27**					
$EC50_{Me2+}$ (nmol/L)	41	580	1639	40	573	1414	37	548	1201	40	612	1286
βMe2+	4.99	2.62	4.49	12.6	2.34	4.40	16.4	2.62	3.06	5.81	2.20	2.97
a		-			**17.3**			-			**2.83**	
AIC		199			190			188			190	
F-test	**F=10.4, p=0.005**						**F=0.15, p=0.71**					

[a] Fitted using the mixture analysis framework of Jonker et al. (2005) as described by Hochmuth et al. (2014);
[b] If $a<0$ the mixture components interact synergistically, if $a>0$ the mixture components interact antagonistically
[c] The F-test compares the nested models CA-non interaction with CA-mixture interaction and the IA-non-interaction with the IA-mixture interaction; $p < 0.05$ indicates significant deviation from non-interactivity. The type of interactive effect can be interpreted from the deviation parameter a[b]
EC50= effective concentration causing 50% effect, β=slope of the concentration response curve, *a*=deviation parameter to quantify mixture interactions, AIC=akaike information criterion

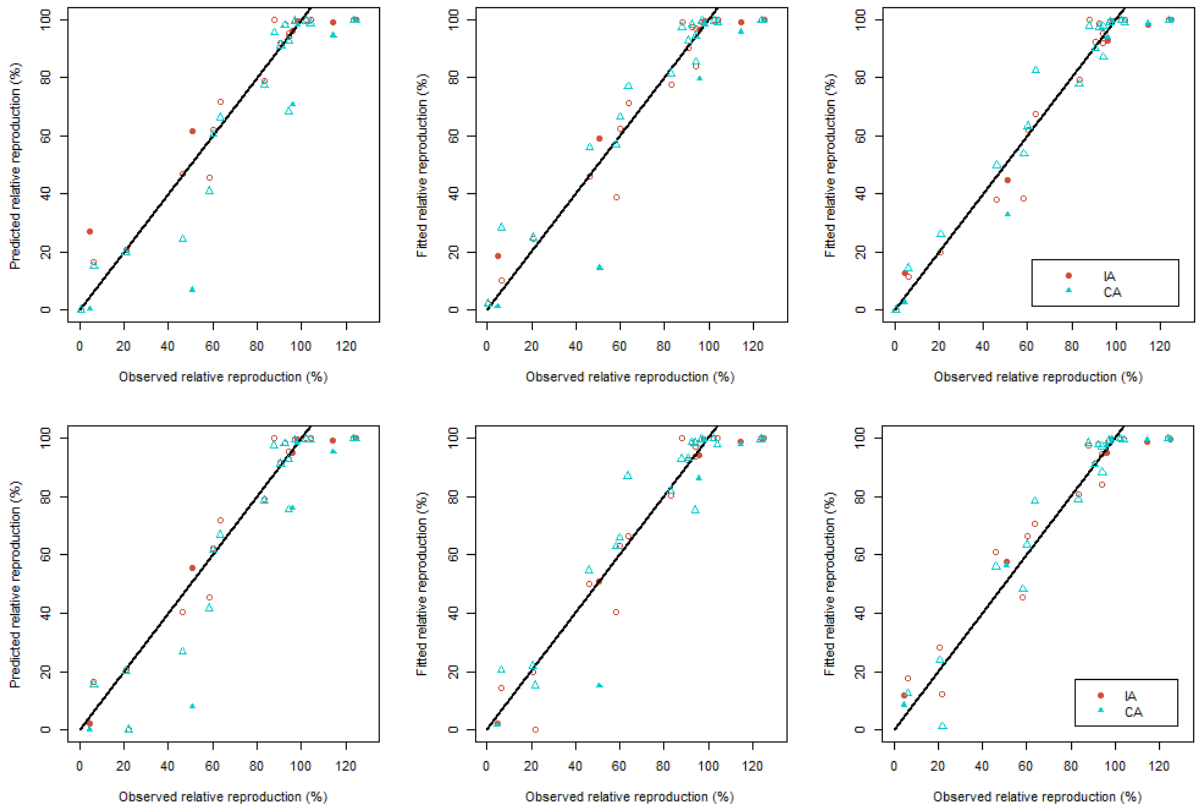

Figure C.7 Observed 7d-relative reproduction of *Ceriodaphnia dubia* (%) versus predicted or fitted relative reproduction (%) for the concentration addition (triangles) and independent action (circles) reference models after exposure to the ternary Ni-Zn-Pb mixture (Expt. 3). Model parameters were based on dissolved concentrations (upper panels) or free ion activities (lower panels). Left panel shows model predictions based on parameters estimated from the single-metal exposures only, middle panels show models fitted to all data (single-metal and mixture treatments), right panel shows model extended with deviation parameter *a* fitted to all data. Open symbols represent data of single metal exposures, filled symbols represent data of mixture treatments.

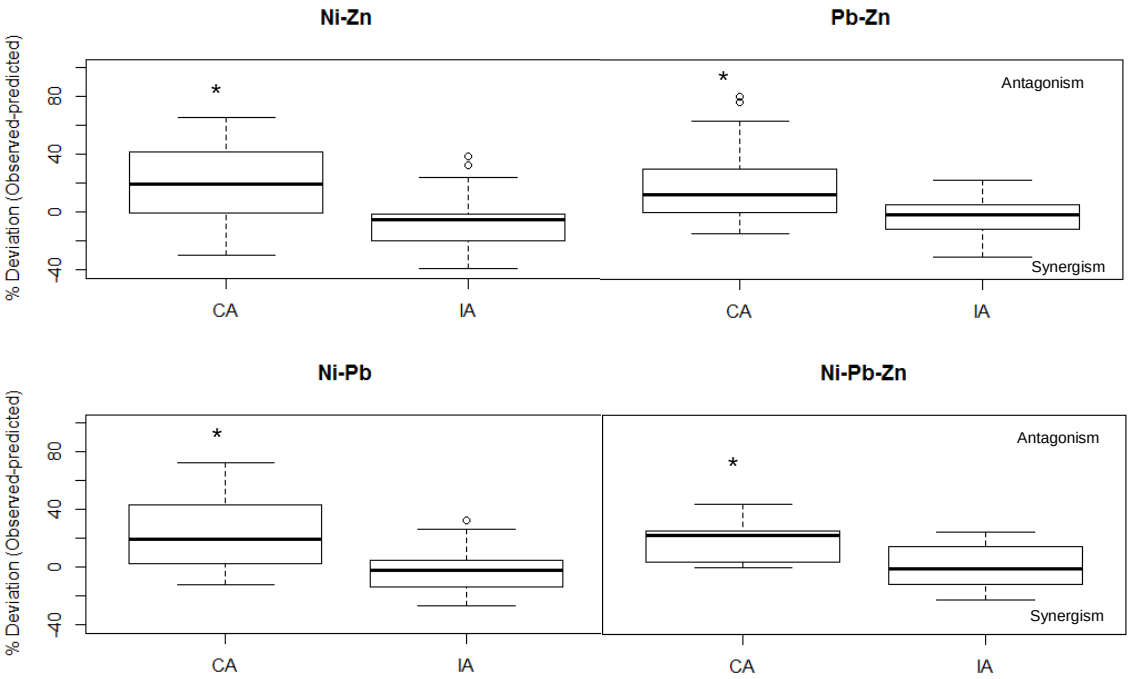

Figure C.8. Deviation (%) between observed 7d-relative reproduction of *Ceriodaphnia dubia* and concentration addition (CA) and independent action (IA) predicted relative reproduction (%) for the different mixtures. Predictions were based on the dissolved concentration parameters ($EC50_{Mediss,i}$ and $\beta_{Mediss,i}$) of the individual concentration-response curves of Ni, Zn, and Pb (Table 6.2). Median values are given in bold, bottom and top of the boxplots give the 25th and 75th percentile. Bottom and top of whiskers represent the 5th and 95th percentile. Circles are outliers. Positive values indicate a trend towards antagonistic deviations, negative values towards synergistic deviation. Asterisks indicate if deviations were significantly different from non-interactivity.

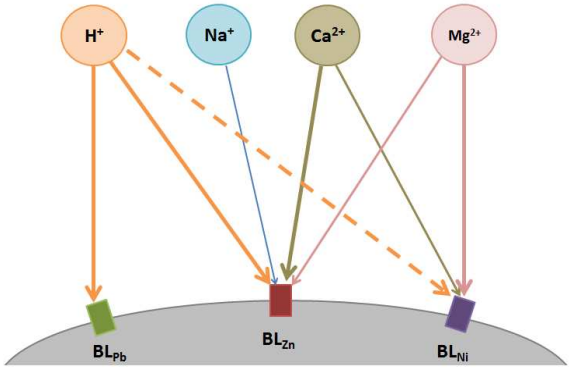

Figure C9.:Visualisation of the chronic daphnid bioavailability models for Pb (Chapter 2), Zn (Chapter 4), and Ni (De Schamphelaere et al. 2006; Deleebeeck et al. 2008). Cations (H^+, Na^+, Ca^{2+} or Mg^{2+}) compete (arrows) with Pb, Zn, or Ni at the respective biotic ligand site (BL_{Pb}, BL_{Zn}, and BL_{Ni}, respectively). The thickness of the arrows is an indication of the relative importance of the competition of a cation relative to each of the metals (e.g. Ca^{2+} and H^+ are the most important competitor at the Zn biotic ligand site), defined by their respective biotic ligand stability constant ($log\ K_{Cat-BL_{Me}}$; see Table 7.2). The dashed line indicates that the effect of pH on Zn and Ni toxicity is not modelled as a single biotic ligand site competition effect, but as a log-linear pH effect superimposed on the competition effects of Ca^{2+}, Mg^{2+} and/or Na^+.

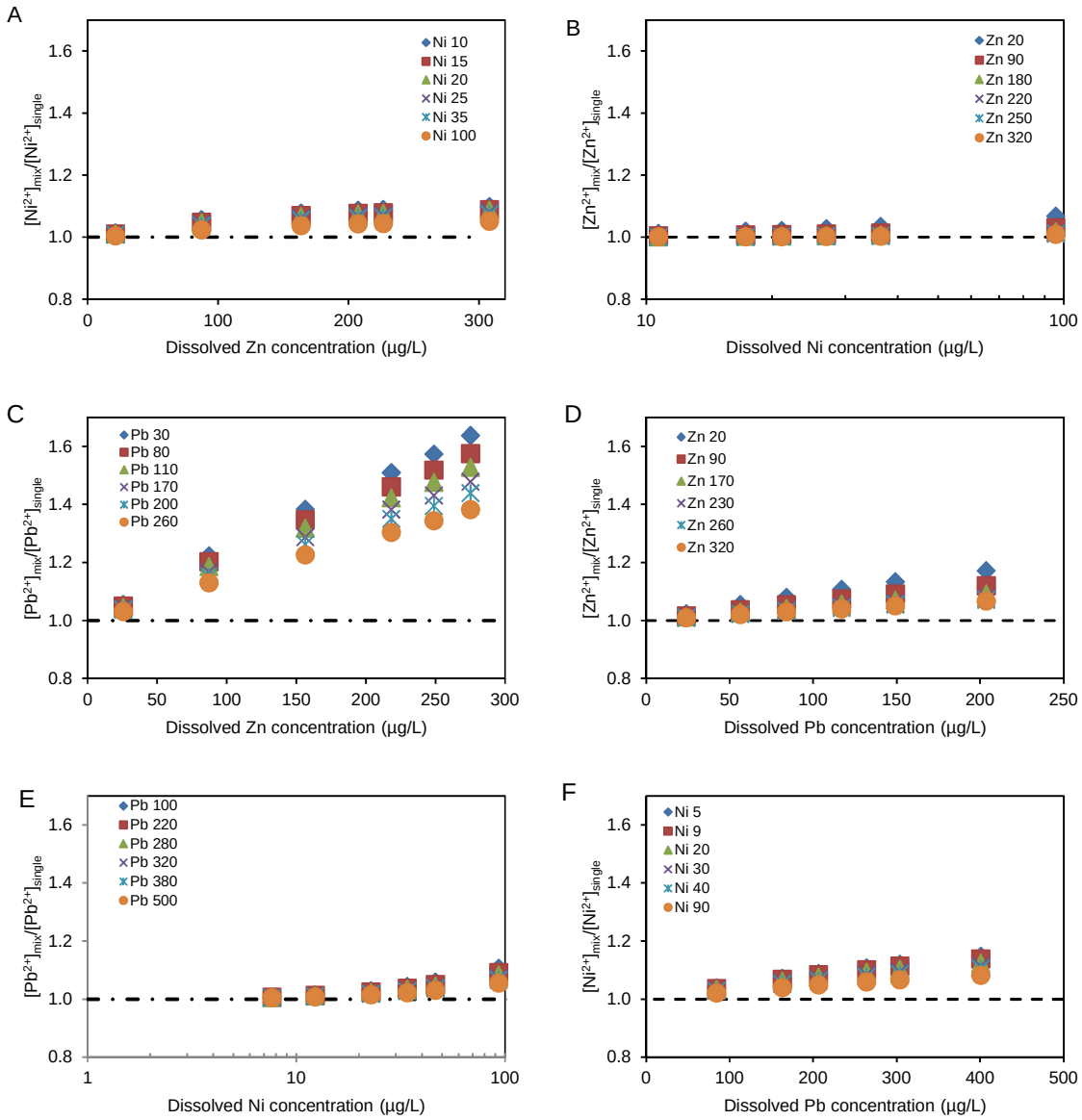

Figure C.10: Ratio of the activity of free Ni^{2+} ions in the mixture treatment ($[Ni^{2+}]_{mix}$) and the activity of free Ni^{2+} ions in the corresponding individual Ni treatment ($[Ni^{2+}]_{single}$) as a function of dissolved Zn concentration (A), ratio of the activity of free Zn^{2+} ions in the mixture treatment ($[Zn^{2+}]_{mix}$) and the activity of free Zn^{2+} ions in the corresponding individual Zn treatment ($[Zn^{2+}]_{single}$) as a function of dissolved Ni concentration (B), ratio of the activity of free Pb^{2+} ions in the mixture treatment ($[Pb^{2+}]_{mix}$) and the activity of free Pb^{2+} ions in the corresponding individual Pb treatment ($[Pb^{2+}]_{single}$) as a function of dissolved Zn concentration (C), ratio of the activity of free Zn^{2+} ions in the mixture treatment ($[Zn^{2+}]_{mix}$) and the activity of free Zn^{2+} ions in the corresponding individual Zn treatment ($[Zn^{2+}]_{single}$) as a function of dissolved Pb concentration (D), ratio of the activity of free Pb^{2+} ions in the mixture treatment ($[Pb^{2+}]_{mix}$) and the activity of free Pb^{2+} ions in the corresponding individual Pb treatment ($[Pb^{2+}]_{single}$) as a function of dissolved Ni concentration (E) and ratio of the activity of free Ni^{2+} ions in the mixture treatment ($[Ni^{2+}]_{mix}$) and the activity of free Ni^{2+} ions in the corresponding individual Ni treatment ($[Ni^{2+}]_{single}$) as a function of dissolved Pb concentration (F). Speciation was modelled as described in the main paper using WHAM VII. The plotted line represents the situation where the activity of free Me^{2+} ions in the mixture is the same as the activity of free Me^{2+} ions in the corresponding individual Me treatment (ratio=1).

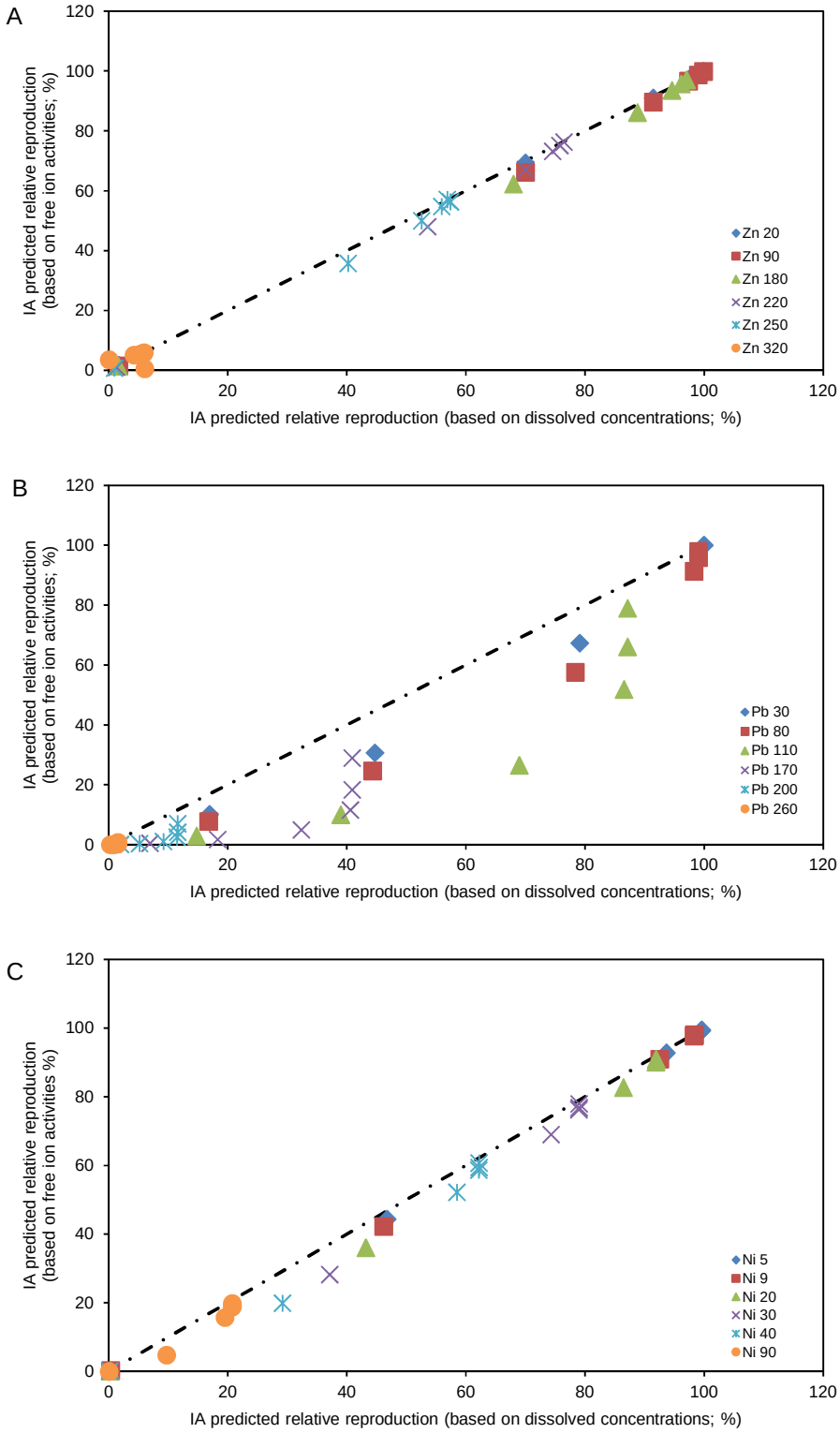

Figure C.11. IA predicted 7d-relative reproduction of *Ceriodaphnia dubia* based on dissolved concentrations plotted against IA predicted relative reproduction as a function of free metal activities for the binary Ni-Zn mixture (A), Pb-Zn mixture (B), and Ni-Pb mixture (C).

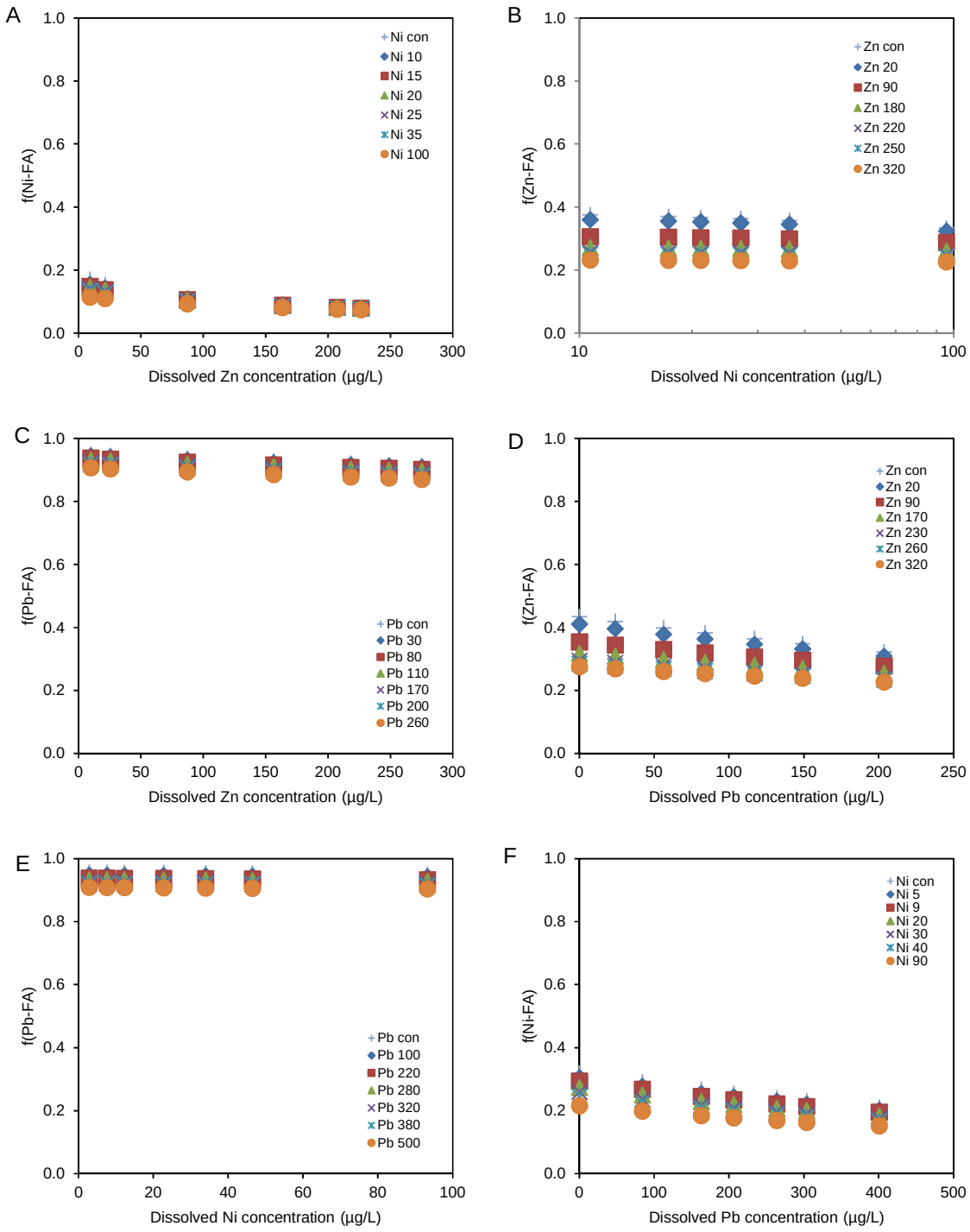

Figure C.12. Fraction of Ni bound to fulvic acid (FA) (f(Ni-FA)) as a function of dissolved Zn concentration (A), fraction of Zn bound toFA (f(Zn-FA)) as a function of dissolved Pb concentration Zn (B), Fraction of Pb bound to fulvic acid (FA) (f(Pb-FA)) as a function of dissolved Zn concentration (C), fraction of Zn bound toFA (f(Zn-FA)) as a function of dissolved Ni concentration Zn (D), Fraction of Pb bound to fulvic acid (FA) (f(Pb-FA)) as a function of dissolved Ni concentration (E), fraction of Pb bound toFA (f(Pb-FA)) as a function of dissolved Ni concentration Zn (F).

Appendix D: Chapter 7: Validation of a metal mixture bioavailability model combining the individual metal biotic ligand models with the independent action model to predict chronic Zn-Ni-Pb mixture toxicity to *Ceriodaphnia dubia*

Table D.1 Control performance of the *Ceriodaphnia dubia* reproduction tests relative to the validation criteria described in the USEPA (2002) test protocol. The results of the Kruskal-Wallis test for inter-plate differences in control reproduction is also listed.

Test id	Number of control replicates	Mean control reproduction (± standard error)[a]	Control mortality (%)[b]	Mothers with 3 broods (%)[c]	Kruskal-Wallis test for inter-plate differences in control reproduction
pH 7	38	21.9±3.6	0	97	p=0.11, χ^2=7.54, df=4
pH 8	42	25.6±4.0	0	98	p=0.71, χ^2=2.12, df=4
Ca 1 mM	45	25.1±5.4	0	96	p=0.69, χ^2=2.26, df=4
Ca 2.5 mM	39	21.4±5.9	5	95	p=0.56, χ^2=3.01, df=4
Brisy	45	22.3±7.1	4	87	p=0.49, χ^2=3.43, df=4
Ankeveen	45	22.1±4.4	2	96	p=0.62, χ^2=2.64, df=4

[a] Control organisms should produce on average at least 15 juveniles (USEPA, 2002)
[b] Control mortality should be less than 20% (USEPA, 2002)
[c] At least 60% of the control organisms should have had 3 broods (USEPA, 2002)

Table D.2. 10%, 20% and 50% 7d-effective concentrations (EC10, EC20, and EC50, respectively, using reproductive toxicity) for *Ceriodaphnia dubia* in the individual Zn, Ni and Pb exposures in the different test waters

Test series	Test ID	$EC50_{Zndiss}$ (µg/L)	$EC20_{Zndiss}$ (µg/L)	$EC10_{Zndiss}$ (µg/L)	$EC50_{Nidiss}$ (µg/L)	$EC20_{Nidiss}$ (µg/L)	$EC10_{Nidiss}$ (µg/L)	$EC50_{Pbdiss}$ (µg/L)	$EC20_{Pbdiss}$ (µg/L)	$EC10_{Pbdiss}$ (µg/L)
pH	Brisy base	225 (198-256)	173 (141-213)	149 (111-199)	39 (33-45)	34 (26-45)	31 (22-45)	135 (115-158)	101 (72-141)	85 (53-137)
	Brisy pH 8	56 (44-71)	31 (20-47)	21 (12-39)	12 (10-14)	8.2 (6.5-10.3)	6.5 (4.7-9.0)	296 (245-359)	206 (163-260)	167 (122-227)
Ca	Brisy base	130 (108-157)	89 (64-123)	71 (46-110)	24 (21-28)	15 (12-19)	11 (8-16)	114 (98-133)	90 (70-114)	78 (57-106)
	Brisy 2 mM	242[a] (177-285)	a	a	30 (26-35)	23 (19-27)	19 (15-24)	171 (155-188)	142 (120-169)	128 (99-165)
DOC	Brisy base	211 (196-228)	184 (168-202)	170 (148-196)	21 (15-29)	9.0 (5.4-15.0)	5.5 (2.7-11.2)	153 (132-178)	118 (82-170)	101 (60-170)
	Ankeveen	145 (133-158)	127 (116-140)	118 (105-133)	23 (20-27)	14 (11-18)	11 (8-15)	312 (293-333)	285 (226-359)	270 (183-398)

[a] Because of the steepness of the dose response, no reliable EC50, EC10, and EC20 could be calculated for Zn for the 2.5 mM Ca test water with the log-logistic dose response. The EC50 was derived from the regression between the observed effect (%) at the 2 concentrations encompassing the 50% effect level and the log filtered concentration. The reported confidence limits for these tests are the 2 concentrations that encompass the EC50.
EC50=median effective concentration; EC20= 20% effective concentration; EC10=10% effective concentration

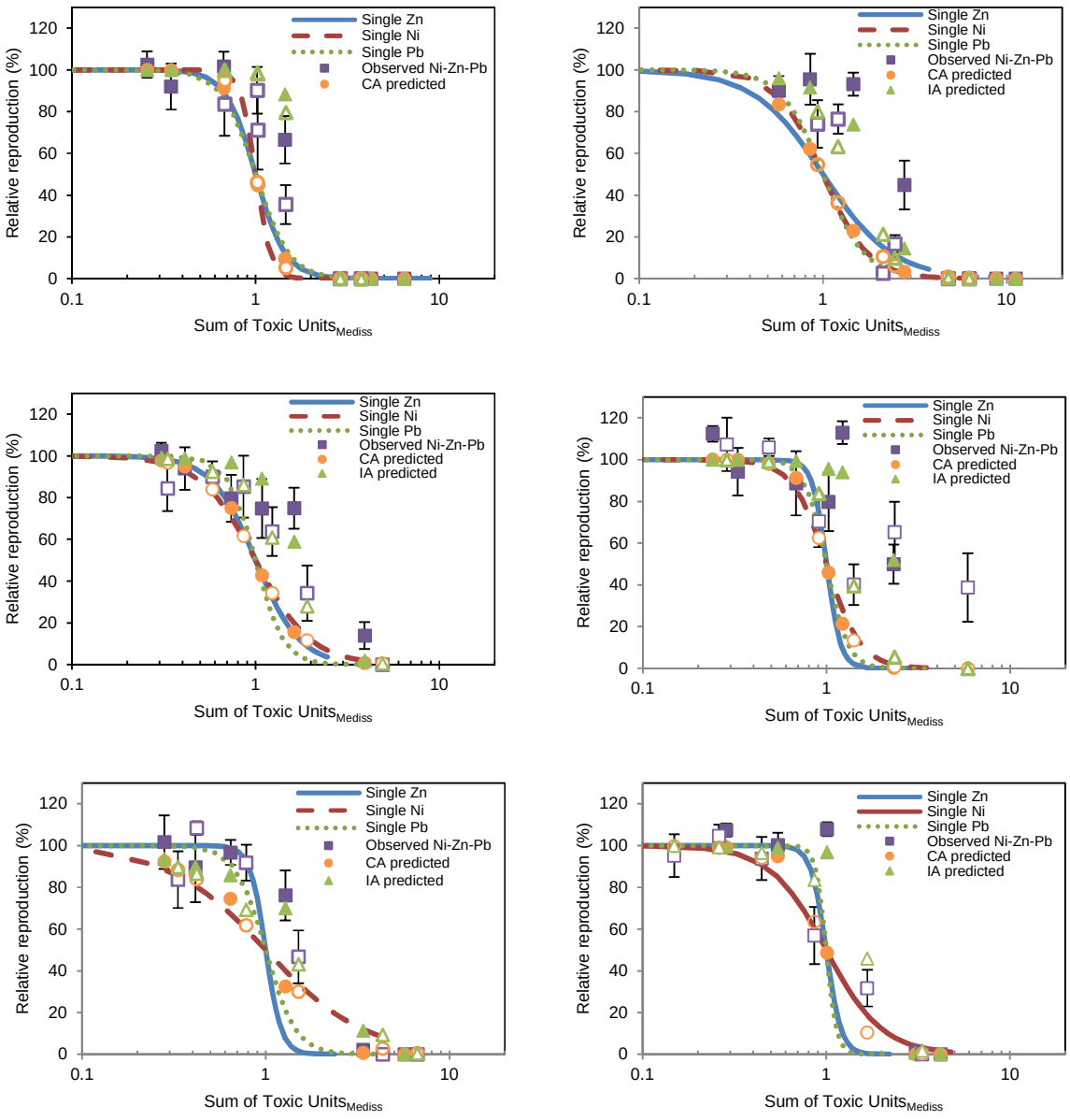

Figure D.1.Observed and predicted 7d-relative reproduction (%) of *Ceriodaphnia dubia* in the mixture combinations of the Ni-Zn-Pb mixture as a function of sum toxic units based on free ion activities in the Brisy pH base (A), Brisy pH 8 (B), Brisy Ca base (C), Brisy Ca 2 mM (D), Brisy nat base (E) and Ankeveen (F) waters. Symbols are denoted as follows: observed reproduction (squares), concentration addition predicted reproduction (circles; equation X), independent action predicted reproduction (triangles; equation X). Filled symbols denote observed and predicted reproduction in the equitoxic mixture ray. Open symbols denote observed and predicted reproduction in the environmental mixture ray. Predictions are based on the parameters ($EC50_{Me2+}$ and β_{Me2+}) of the individual concentration-response curves of Ni, Zn, and Pb (Equation 3). Lines represent the individual log-logistic dose response curve (Equation 3) of Ni (dashed line), Zn (full line), and Pb (dotted line) in the corresponding test waters.

Table D.3 Estimated model parameters for the mixture reference models Concentration Addition (CA; Equation 7.4) and Independent Action (IA; Equation 7.5)[a] in the Brisy pH base water

Test id		CA non-interaction			CA mixture interaction			IA non-interaction			IA mixture interaction		
		Zn	**Ni**	**Pb**	**Zn**	**Ni**	**Pb**	**Zn**	**Ni**	**Pb**	**Zn**	**Ni**	**Pb**
Equitoxic ray	**$EC50_{Mediss}$ (µg/L)**	260	45	173	240	37	14	227	39	129	221	38	143
	β_{Mediss}	4.40	5.64	3.72	4.57	6.42	4.03	5.06	10.5	3.57	4.87	10.5	6.29
	a^b		-			**13**			-			**-38**	
	AIC		182			166			161			160	
	F-test		**F=18, p<0.001**						**F=2.3, p=0.15**				
	$EC50_{Me2+}$ (µg/L)	1629	367	21	1586	328	15	1393	348	15	1439	350	16
	β_{Me2+}	3.85	6.15	2.24	3.88	5.68	2.62	3.98	9.18	2.93	3.96	10.8	3.98
	a^b		-			**21**			-			**-19**	
	AIC		179			168			159			159	
	F-test		**F=12, p=0.004**						**F=1.1, p=0.32**				
Environmental ray	**$EC50_{Mediss}$ (µg/L)**	246	47	155	226	41	111	212	32	136	243	32	117
	β_{Mediss}	4.72	6.32	4.55	6.71	5.31	3.26	2.98	13.9	5.95	3.39	22.0	6.67
	a^b		-			**115**			-			**-3**	
	AIC		177			171			171			171	
	F-test		**F=6.3, p=0.02**						**F=2.1, p=0.17**				
	$EC50_{Me2+}$ (µg/L)	1626	393	17	1478	348	16	1334	315	15	1305	305	17
	β_{Me2+}	4.74	5.50	2.82	5.13	4.17	3.48	3.41	6.79	2.71	3.86	5.88	5.57
	a^b		-			**135**			-			**-76**	
	AIC		176			174			170			169	
	F-test		**F=3.0, p=0.11**						**F=2.0, p=0.18**				

[a] Fitted using the mixture analysis framework of Jonker et al. (2005) as described by Hochmuth et al. (2014);
[b] If a<0 the mixture components interact synergistically, if a>0 the mixture components interact antagonistically
[c] The F-test compares the nested models CA-non interaction with CA-mixture interaction and the IA-non-interaction with the IA-mixture interaction; p < 0.05 indicates significant deviation from non-interactivity. The type of interactive effect can be interpreted from the deviation parameter a^b
EC50= effective concentration causing 50% effect, β=slope of the dose response curve, a=deviation parameter to quantify mixture interactions, AIC=akaike information criterion

Table D.4. Estimated model parameters for the mixture reference models Concentration Addition (CA; Equation 7.4) and Independent Action (IA; Equation 7.5)[a] in the Brisy pH 8 water

Test id		CA non-interaction			CA mixture interaction			IA non-interaction			IA mixture interaction		
		Zn	**Ni**	**Pb**	**Zn**	**Ni**	**Pb**	**Zn**	**Ni**	**Pb**	**Zn**	**Ni**	**Pb**
Equitoxic ray	**$EC50_{Mediss}$ (µg/L)**	80	16	435	65	15	289	56	14	342	48	13	284
	β_{Mediss}	2.31	3.71	3.83	2.17	3.53	4.18	2.12	4.28	4.40	1.78	3.75	3.68
	a^b		-			**26**			-			**24**	
	AIC		189			153			163			153	
	F-test		**F=75, p<0.001**						**F=11, p=0.006**				
	$EC50_{Me2+}$ (µg/L)	245	110	18	250	90	9.9	222	84	13	192	82	8
	β_{Me2+}	1.76	3.71	3.18	2.21	3.64	3.31	2.49	3.86	3.63	2.10	3.67	2.58
	a^b		-			**29**						**40**	
	AIC		187			151			167			152	
	F-test		**F=71, p<0.001**						**F=17, p=0.001**				
Environmental ray	**$EC50_{Mediss}$ (µg/L)**	69	16	407	71	16	309	51	13	290	55	12	351
	β_{Mediss}	2.67	4.08	5.30	3.25	3.45	4.50	2.23	4.18	4.03	2.22	3.95	4.69
	a^b		-			**15**			-			**-23**	
	AIC		162			164			158			159	
	F-test		**F=0, p=1.00**						**F=0.7, p=0.41**				
	$EC50_{Me2+}$ (µg/L)	274	93	15	252	90	9	185	76	10	176	76	11
	β_{Me2+}	2.44	3.81	4.33	2.56	3.72	2.64	1.74	4.99	3.55	2.05	3.96	3.33
	a^b		-			**36**			-			**-0.1**	
	AIC		161			162			155			158	
	F-test		**F=0.17, p=0.69**						**F=-0.4, p=1.00**				

[a] Fitted using the mixture analysis framework of Jonker et al. (2005) as described by Hochmuth et al. (2014);
[b] If a<0 the mixture components interact synergistically, if a>0 the mixture components interact antagonistically
[c] The F-test compares the nested models CA-non interaction with CA-mixture interaction and the IA-non-interaction with the IA-mixture interaction; p < 0.05 indicates significant deviation from non-interactivity. The type of interactive effect can be interpreted from the deviation parameter a^b
EC50= effective concentration causing 50% effect, β=slope of the dose ponse curve, a=deviation parameter to quantify mixture interactions, AIC=akaike information criterion

Table D.5. Estimated model parameters for the mixture reference models Concentration Addition (CA; Equation 7.4) and Independent Action (IA; Equation 7.5)[a] in the Brisy Ca base water

Test id		CA non-interaction			CA mixture interaction			IA non-interaction			IA mixture interaction		
		Zn	Ni	Pb	Zn	Ni	Pb	Zn	Ni	Pb	Zn	Ni	Pb
Equitoxic ray	$EC50_{Mediss}$ (µg/L)	159	34	131	145	25	124	135	25	119	134	26	119
	β_{Mediss}	3.29	2.47	7.02	4.40	2.23	6.70	3.64	2.42	25.1	4.41	2.57	6.27
	a[b]	-			**22**			-			**-2**		
	AIC	180			161			159			161		
	F-test	**F=25, p<0.001**						**F=-0.3, p=1.00**					
	$EC50_{Me2+}$ (µg/L)	898	253	14	817	230	11	799	205	11	722	217	11
	β_{Me2+}	2.79	2.39	6.17	3.51	2.54	4.94	4.43	2.22	4.63	2.88	2.28	3.98
	a[b]	-			**24**			-			**16**		
	AIC	180			160			160			162		
	F-test	**F=25; p<0.001**						**F=-0.05; p=1.00**					
Environmental ray	$EC50_{Mediss}$ (µg/L)	147	30	134	140	27	98	134	25	112	129	25	109
	β_{Mediss}	4.23	2.40	7.32	3.65	3.00	3.46	4.38	2.56	5.81	3.70	2.45	5.48
	a[b]	-			**103**			-			**40**		
	AIC	160			155			143			145		
	F-test	**F=5.4, p=0.04**						**F=0, p=1.00**					
	$EC50_{Me2+}$ (µg/L)	832	262	13	771	233	9.0	752	216	11	811	209	8.27
	β_{Me2+}	4.17	2.33	6.23	3.88	2.67	3.94	3.26	2.62	4.67	4.36	2.32	4.62
	a[b]	-			**105**			-			**-54**		
	AIC	159			154			143			146		
	F-test	**F=5.6, p=0.03**						**F=-0.6, p=1.00**					

[a] Fitted using the mixture analysis framework of Jonker et al. (2005) as described by Hochmuth et al. (2014);
[b] If *a*<0 the mixture components interact synergistically, if *a*>0 the mixture components interact antagonistically
[c] The F-test compares the nested models CA-non interaction with CA-mixture interaction and the IA-non-interaction with the IA-mixture interaction; p < 0.05 indicates significant deviation from non-interactivity. The type of interactive effect can be interpreted from the deviation parameter a^b
EC50= effective concentration causing 50% effect, β=slope of the dose response curve, *a*=deviation parameter to quantify mixture interactions, AIC=akaike information criterion

Table D.6 Estimated model parameters for the mixture reference models Concentration Addition (CA; Equation 7.4) and Independent Action (IA; Equation 7.5)[a] in the Brisy Ca 2mM water

Test id		CA non-interaction			CA mixture interaction			IA non-interaction			IA mixture interaction		
		Zn	Ni	Pb	Zn	Ni	Pb	Zn	Ni	Pb	Zn	Ni	Pb
Equitoxic ray	$EC50_{Mediss}$ (µg/L)	284	40	190	278	33	187	263	31	171	263	30	170
	β_{Mediss}	9.71	3.63	8.58	9.09	3.67	9.51	15.7	5.03	7.51	14.1	3.89	8.20
	a[b]	-			**21**			-			**7**		
	AIC	199			171			165			168		
	F-test	**F=45, p<0.001**						**F=-0.8, p=1.00**					
	$EC50_{Me2+}$ (µg/L)	1875	374	36	1813	308	34	1749	288	30	1742	279	30
	β_{Me2+}	7.50	3.89	5.13	8.08	3.60	8.72	15.0	5.22	7.90	13.3	5.12	6.35
	a[b]	-			**21**			-			**5**		
	AIC	198			173			165			166		
	F-test	**F=37, p<0.001**						**F=0.9, p=0.37**					
Environmental ray	$EC50_{Mediss}$ (µg/L)	266	38	182	276	36	190	249	34	174	252	36	172
	β_{Mediss}	8.12	3.00	10.2	9.52	3.32	7.29	10.6	2.51	6.75	9.00	2.33	7.72
	a[b]	-			**-15**			-			**-16**		
	AIC	194			196			188			190		
	F-test	**F=-0.2, p=1.00**						**F=0, p=1.00**					
	$EC50_{Me2+}$ (µg/L)	1857	325	34	1904	290	32	1722	289	31	1796	299	29
	β_{Me2+}	8.25	2.44	4.43	8.19	2.71	5.92	8.91	2.07	5.53	10.4	2.60	6.74
	a[b]	-			**35**			-			**10**		
	AIC	194			196-			189			194		
	F-test	**F=-0.3, p=1.00**						**F=-0.1, p=1.00**					

[a] Fitted using the mixture analysis framework of Jonker et al. (2005) as described by Hochmuth et al. (2014);
[b] If *a*<0 the mixture components interact synergistically, if *a*>0 the mixture components interact antagonistically
[c] The F-test compares the nested models CA-non interaction with CA-mixture interaction and the IA-non-interaction with the IA-mixture interaction; p < 0.05 indicates significant deviation from non-interactivity. The type of interactive effect can be interpreted from the deviation parameter a^b
EC50= effective concentration causing 50% effect, β=slope of the dose response curve, *a*=deviation parameter to quantify mixture interactions, AIC=akaike information criterion

Table D.7 Estimated model parameters for the mixture reference models Concentration Addition (CA; Equation 7.4) and Independent Action (IA; Equation 7.5)[a] in the Brisy nat base water

Test id		CA non-interaction			CA mixture interaction			IA non-interaction			IA mixture interaction		
		Zn	**Ni**	**Pb**	**Zn**	**Ni**	**Pb**	**Zn**	**Ni**	**Pb**	**Zn**	**Ni**	**Pb**
Equitoxic ray	**$EC50_{Mediss}$ (µg/L)**	241	31	172	225	21	167	204	22	153	210	21	153
	β_{Mediss}	9.20	1.71	5.06	15.9	1.78	7.56	17.9	1.95	4.72	15.4	2.08	5.41
	a^b		-			**17**			-			**-3**	
	AIC		172			153			151			155	
	F-test		**F=22, p<0.001**						**F=1.2, p=1.00**				
	$EC50_{Me2+}$ (µg/L)	1505	252	24	1532	205	20	1334	185	20	1346	176	19
	β_{Me2+}	8.86	1.84	3.42	13.0	1.62	4.13	10.9	1.85	5.29	10.8	1.73	4.29
	a^b		-			**19**			-			**14**	
	AIC		170			154			150			152	
	F-test		**F=20, p<0.001**						**F=0.2, p=0.66**				
Environmental ray	**$EC50_{Mediss}$ (µg/L)**	214	23	157	213	22	152	236	26	172	229	29	144
	β_{Mediss}	11.3	2.40	5.47	9.30	2.41	5.86	18.0	2.01	6.51	11.0	2.14	7.17
	a^b		-			**-43**			-			**117**	
	AIC		162			164			164			166	
	F-test		**F=-0.1, p=1.00**						**F=0.3, p=0.59**				
	$EC50_{Me2+}$ (µg/L)	1455	246	21	1457	240	20	1348	213	20	1347	213	19
	β_{Me2+}	13.5	1.89	4.16	14.1	2.06	5.86	9.12	2.21	5.83	9.27	2.12	4.13
	a^b		-			**99**			-			**-34**	
	AIC		164			167			162			163	
	F-test		**F=-0.2, p=1.00**						**F=0.5, p=0.51**				

[a] Fitted using the mixture analysis framework of Jonker et al. (2005) as described by Hochmuth et al. (2014);
[b] If $a<0$ the mixture components interact synergistically, if $a>0$ the mixture components interact antagonistically
[c] The F-test compares the nested models CA-non interaction with CA-mixture interaction and the IA-non-interaction with the IA-mixture interaction; $p < 0.05$ indicates significant deviation from non-interactivity. The type of interactive effect can be interpreted from the deviation parameter a^b
EC50= effective concentration causing 50% effect, β=slope of the dose response curve, *a*=deviation parameter to quantify mixture interactions, AIC=akaike information criterion

Table D.8 Estimated model parameters for the mixture reference models Concentration Addition (CA; Equation 4) and Independent Action (IA; Equation 5)[a] in the Ankeveen water

Test id		CA non-interaction			CA mixture interaction			IA non-interaction			IA mixture interaction		
		Zn	**Ni**	**Pb**	**Zn**	**Ni**	**Pb**	**Zn**	**Ni**	**Pb**	**Zn**	**Ni**	**Pb**
Equitoxic ray	**$EC50_{Mediss}$ (µg/L)**	163	27	347	145	24	324	147	23	312	144	23	310
	β_{Mediss}	6.90	3.08	5.92	10.9	2.95	10.2	11.0	3.37	20.2	9.70	3.19	20.2
	a^b		-			**20**			-			**32**	
	AIC		174			145			139			140	
	F-test		**F=48, p<0.001**						**F=0.8, p=0.38**				
	$EC50_{Me2+}$ (µg/L)	533	171	10	520	130	9	470	122	9	470	120	8.64
	β_{Me2+}	5.41	2.89	4.80	11.0	3.21	7.63	8.34	3.10	16.2	9.86	2.86	17.6
	a^b					**27**			-			**56**	
	AIC		173			144			140			140	
	F-test		**F=50, p<0.001**						**F=1.8, p=0.20**				
Environmental ray	**$EC50_{Mediss}$ (µg/L)**	150	24	343	152	23	318	143	19	314	137	20	317
	β_{Mediss}	12.4	2.21	11.5	13.2	2.28	9.81	10.3	2.47	12.7	17.2	2.48	14.4
	a^b		-		**54**				-			**-92**	
	AIC		154			155			156			154	
	F-test		**F=0.7, p=0.40**						**F=2.3, p=0.15**				
	$EC50_{Me2+}$ (µg/L)	510	135	9	513	141	10	477	115	9	490	117	9
	β_{Me2+}	8.92	2.20	5.84	10.7	2.13	7.23	10.1	2.44	13.9	9.19	2.63	15.5
	a^b		-			**-84**			-			**-119**	
	AIC		155			155			151			150	
	F-test		**F=1.3, p=0.28**						**F=2.7, p=0.12**				

[a] Fitted using the mixture analysis framework of Jonker et al. (2005) as described by Hochmuth et al. (2014);
[b] If $a<0$ the mixture components interact synergistically, if $a>0$ the mixture components interact antagonistically
[c] The F-test compares the nested models CA-non interaction with CA-mixture interaction and the IA-non-interaction with the IA-mixture interaction; $p < 0.05$ indicates significant deviation from non-interactivity. The type of interactive effect can be interpreted from the deviation parameter a^b
EC50= effective concentration causing 50% effect, β=slope of the dose response curve, *a*=deviation parameter to quantify mixture interactions, AIC=akaike information criterion

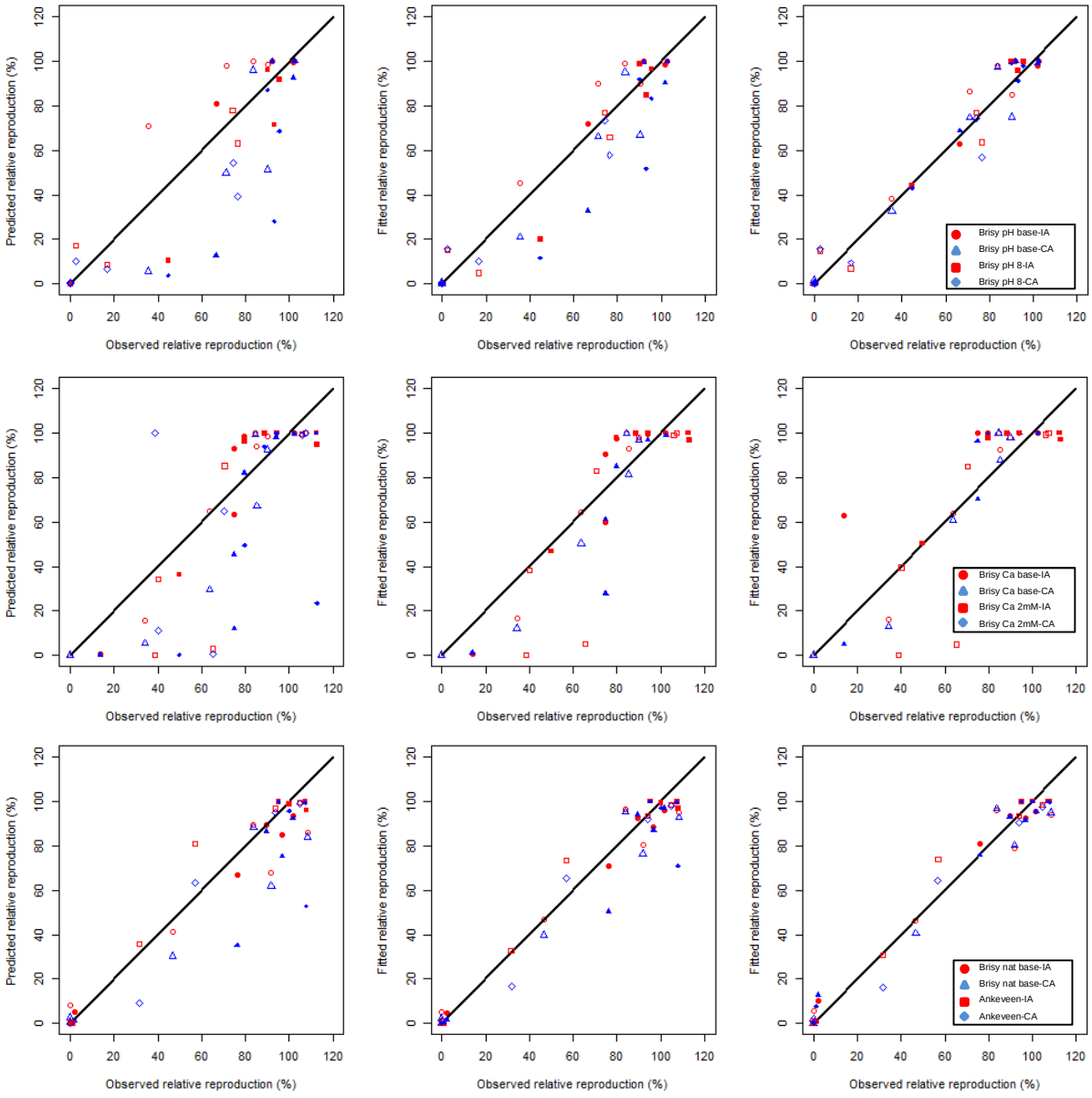

Figure D.2 Observed 7d-relative reproduction (%) of *Ceriodaphnia dubia* versus predicted or fitted relative reproduction (%) for the concentration addition (circles, squares) and independent action (triangles, diamonds) reference models after exposure to the ternary Ni-Zn-Pb mixtures in the Brisy pH series (upper panels), Brisy Ca series (middle panels) and the natural water series (lower panels). Model parameters were based on free ion activities. Left panel shows model predictions based on parameters estimated from the single-metal exposures alone (Table 7.3), middle panels show models fitted to all data (single-metal and mixture treatments; parameters reported in Table D.3-8), right panel shows model extended with deviation parameter *a* fitted to all data (single-metal and mixture treatments; parameters reported in Table D.3-8). Filled symbols represent data of the equitoxic ray, open symbols represent data of the environmental ray.

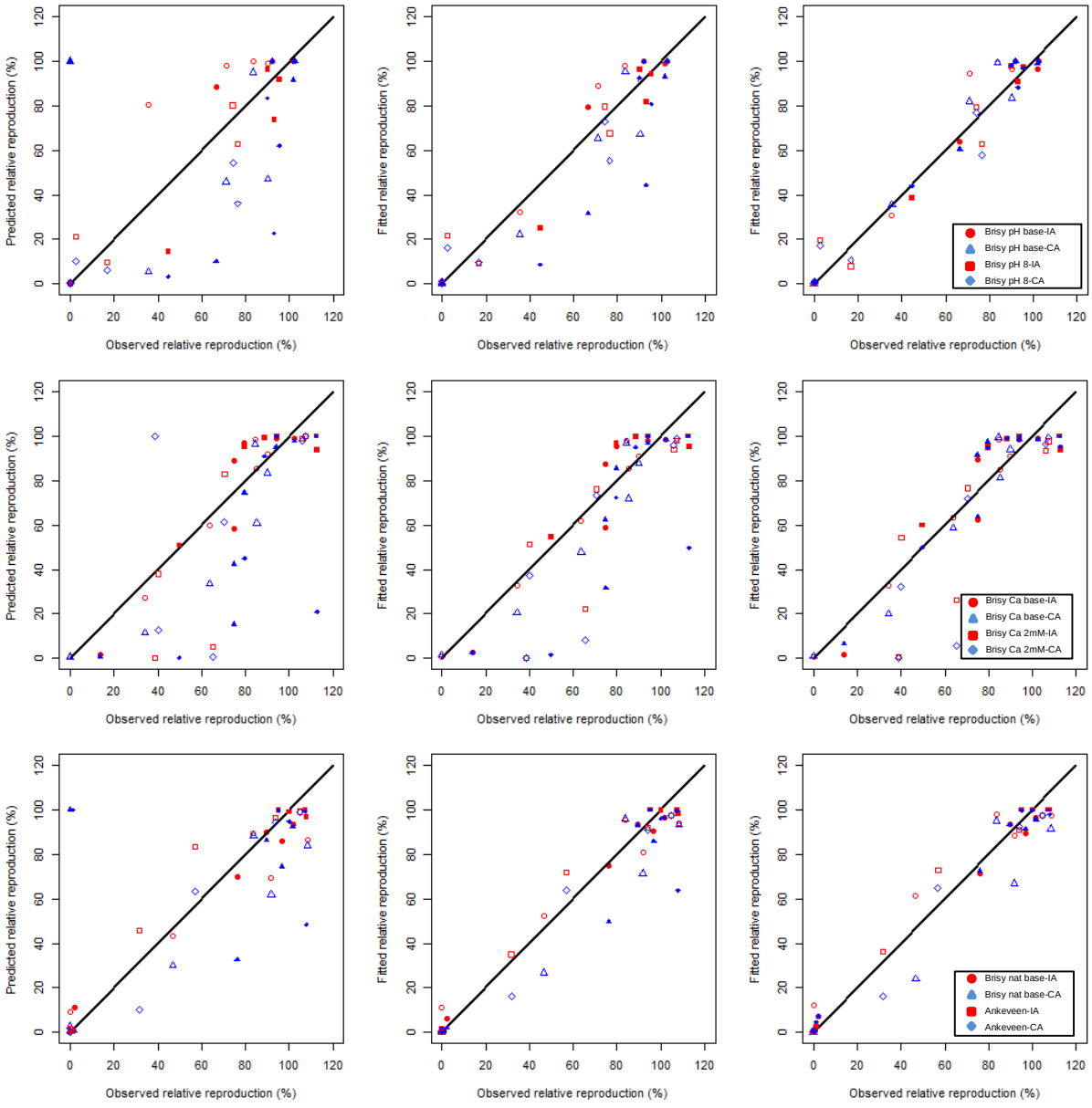

Figure D.3 Observed 7d-relative reproduction (%) of *Ceriodaphnia dubia* versus predicted or fitted relative reproduction (%) for the concentration addition (circles, squares) and independent action (triangles, diamonds) reference models after exposure to the ternary Ni-Zn-Pb mixtures in the Brisy pH series (upper panels), Brisy Ca series (middle panels) and the natural water series (lower panels). Model parameters were based on dissolved metal concentrations. Left panel shows model predictions based on parameters estimated from the single-metal exposures alone (Table 7.3), middle panels show models fitted to all data (single-metal and mixture treatments; parameters reported in Table D.3-8), right panel shows model extended with deviation parameter *a* fitted to all data (single-metal and mixture treatments; parameters reported in Table D.3-8). Filled symbols represent data of the equitoxic ray, open symbols represent data of the environmental ray.

Appendix E: Chapter 8: Chronic metal mixture toxicity to *Ceriodaphnia dubia*: implications for ecological risk assessment

Data of measured dissolved Ni, Zn, and Pb concentrations were obtained from the Dommel monitoring databases. Only measurements conducted in 2010 were considered. Data were only retained if the most important water-chemistry variables, i.e. DOC (dissolved organic carbon), Ca, pH, and dissolved concentrations of Ni, Zn, and Pb, were reported. If dissolved concentrations were below the detection limit, the detection limit divided by 2 was used in the analysis. When concentrations of other major ions (Mg, K, Na, Cl, SO_4) were not reported, they were calculated based on reported regression relations between Ca & major ions for EU water bodies (Van Sprang et al. 2009). Inorganic carbon concentrations were derived from reported pH and alkalinity (Van Sprang et al. 2009).

Bioavailability normalizations of toxicity data in the toxicity databases of the individual metals were conducted using the species sensitivity distribution (SSD) bioavailability normalization approach applied in the European risk assessment frameworks for these metals (DEPA 2008; Van Sprang et al. 2009; 2016). For Ni, bioavailability normalisations were conducted using the 'chronic Ni bioavailability and normalization tool' reported by Nys et al. (2016). For Pb, bioavailability normalisations of the Pb toxicity database were performed using the 'Lead BLM SSD normalization tool' (Van Sprang et al. 2016). For Zn, bioavailability normalization were performed following the approach reported by Van Sprang et al. (2009) using the chronic Zn toxicity database updated by Van Regenmortel et al. (2014). HC5, i.e. median estimates of the HC5, for every metal and $PAF_{Mix,IA\text{-}SSD}$ were derived from the normalised toxicity data using the log-normal species sensitivity distribution (Aldenberg and Jaworska 2000).

In the CA-DRC, only the species which are shared between the different toxicity databases of the individual metals were considered. Following 9 species are shared between the chronic toxicity databases of Ni, Zn, and Pb: *Brachionus calyciflorus, C. dubią Chlorella sp., D. magna, Hyalella azteca, Lymneae stagnalis, Oncorhynchus mykiss, Pimephales promelas,* and *Pseudokirchneriella subcapitata*. PAF_{Mix} in the CA-DRC methods was calculated by fitting a normal-distribution function to the bioavailbility normalized TU_{EC10} of these species.